TO GROW AND SURVIVE OR REPRODUCE AND DIE? LIFE-HISTORY STRATEGIES AND ECOLOGICAL INTERACTIONS BETWEEN THE MUSSELS *Mytilus edulis* (LINNAEUS, 1758) AND *Mytilus trossulus* (GOULD, 1850) IN THE NORTHWEST ATLANTIC

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A thesis submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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September, 2008

St. John's Newfoundland

ABSTRACT

The spatial and temporal distributions of two closely related blue mussel species (*Mytilus edulis* and *M. trossulus*) which co-exist within a mosaic hybrid zone in Newfoundland are not well understood. Where closely related species do not clearly differentiate along resource or physiological gradients, resource allocation to traits that enhance their ecological differentiation in fluctuating environments could stabilize their co-existence. Accordingly, a key objective of this study was to relate the plasticity of growth (which influences future survival), reproductive effort (which influences dispersal and colonization), and survival (linked to maintenance), among *M. edulis*, *M. trossulus* and their reciprocal F1 hybrids to variation in site productivity. The findings determined the design of a subsequent series of experiments, which focussed on predator inducible defenses and density dependent intra-specific and inter-specific interactions between *M. edulis* and *M. trossulus*. Reproductive cycles were generally similar among the parental species and F1 hybrids. Gamete output was significantly higher in *M. trossulus* than in *M. edulis* and the F1 hybrids. *M. edulis* prioritized allocation of resources to somatic traits (growth, shell thickness and adductor mass) to a greater extent than *M. trossulus,* while *M. trossulus* prioritized allocation to reproduction (reproductive effort) to a greater degree than *M. edulis*. As in *M. trossulus*, but not in *M. edulis*, growth among sites in both hybrid groups did not change. Flexible allocation to reproduction among sites was evident in the F1 hybrids, but relative to *M. trossulus* overall reproductive effort was considerably lower. Hybrid *edulis* (*M. edulis* female parent) survival was also lower than in both parental species, while hybrid *trossulus* (*M. trossulus* female parent) survival was not significantly different, although variation was greater in hybrid *trossulus*. These attributes likely reduce the establishment success of the F1 hybrid groups. In an experiment on predator induced defensive allocation, *M. edulis* also developed a thicker shell and heavier adductor muscle than *M. trossulus* in the presence of sea stars, and a thicker shell than *M. trossulus* in the presence of crabs. Sea stars and crabs also induced stronger byssal attachment in both species, albeit to a greater extent in *M. edulis* than *M.*

trossulus. Compared with *M. trossulus*, *M. edulis* growth decreased at a much greater rate with increasing density. Given that food availability (if limited) and/or space may increase with decreasing density, these findings provide additional evidence that *M. edulis* prioritizes allocation to somatic traits to a greater extent than *M. trossulus*. *M. edulis* also grew faster in the presence of *M. trossulus* than when grown with an equal number of *M. edulis* only. Survival of *M. edulis* and *M. trossulus* was not affected by density, regardless of whether the mussels were maintained in the presence or absence of the other species. In conclusion, faster growth resulting from increased space availability or site productivity, or from the presence of *M. trossulus,* together with enhanced defenses in the presence of predators, increases the resistance of *M. edulis* to biotic disturbance and therefore its likelihood of displacing *M. trossulus*. Conversely, *M. trossulus* invests more resources to reproduction relative to *M. edulis*, which likely increases its ability to re-colonize disturbed environments. Inter-specific differences in colonization or displacement abilities in disturbed environments reinforce stabilizing and equalizing mechanisms maintaining species co-existence. Resource allocation trade-offs and associated ecological differentiation in disturbed environments likely play an important and overlooked role in maintaining species diversity among assemblages of closely related interbreeding sessile species.

Acknowledgements

I would like to express my sincere gratitude to my supervisors Dr. David Innes and Dr. Raymond Thompson for their guidance and support while writing the thesis. I would also like to thank Dr. Patrick Dabinett for his support early on in the production of the thesis. I thank Guangxu Liu, Yusuke Kosewik, and Marcelo Miranda for their friendship, patience, and feedback. Additionally I would like to thank Marcelo Miranda for his role in the laboratory production of *Mytilus edulis*, *M. trossulus* and their F1 hybrids, approximately 4000 in total of which were used in this thesis. The mussel farmers Richard Pippy, and Joe Keating provided essential logistical support, without which much of the fieldwork could not have been accomplished. Erin Stapleton, Brad Butler, Krista Benson, Chelsea Heigns and April Penney all provided valuable assistance with the laboratory work at both the Biology department and the Ocean Science Center. Without their help, all the samples could not have been processed and analyzed. I would also like to thank the personnel of the Marine Institute of MUN, specifically Cyr Couturier, Jason Nichols, Alistair Struthers and Sean Macneill for assistance with field sampling and advice on mussel cultivation. Financial support was provided by grants to Dr. David Innes and Dr. Raymond Thompson from the Natural Sciences and Engineering Council (NSERC), AquaNet (one of the Networks of Centres of Excellence funded by the Government of Canada), and a graduate scholarship from Memorial University.

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List of Abbreviations

Anova = Analysis of Variance
Ancova = Analysis of covariance
BSC = Biological Species Concept
CC=Competition/colonization hypothesis
E = *Mytilus edulis*
ESS= Evolutionary Stable Strategy
HE = F1 hybrids from *Mytilus edulis* female and *Mytilus trossulus* male
F= F ratio
F1 = First generation
FSW = Fresh seawater
GDC: Growth density covariance (GDC)
GLU = Polyphenolic adhesive protein marker
GLM= General Linear Model
h = Hours
JN= Johnson Neyman Procedure
LH = Long Harbour, Placentia Bay.
m = Meters
mg = Milligrams
min = Minutes
ml = Milliliters
mm = Millimeters
N = Number (sample size)
OC = Celsius degrees
PCR = Polymerase chain reaction
ppt = Parts per thousand
P<n= Probability
R= R Project for Statistical Computing
s = Seconds
SD = Standard deviation
SN= Successional niche
SSE= Spatial storage effect
STD= Standardised
T = *Mytilus trossulus*
HT = F1 hybrid from *Mytilus trossulus* female and *Mytilus edulis* male
TR= Trinity, Trinity Bay.
μm = Micrometres
UV = Ultra Violet
V = Volume

Chapter 1

General Introduction

1.1. The development and application of life-history theory

The publication of the "Origin of Species" by Charles Darwin in 1859 demonstrated that descent with modification and natural selection could explain many facets of biology. By the middle of the twentieth century the general mechanism of inheritance had been resolved, although there remained considerable uncertainty regarding how genetics and natural selection could explain the evolution of whole organism traits or phenotypes (Stearns 2000). The evolution of phenotypes in the context of an organism's environment then became the central focus of life-history theorists, who sought to explain how evolution shapes organisms to achieve reproductive success (Stearns 2000).

Given the wealth of life-history patterns, life-history theorists faced a daunting task. Optimal age at maturity, reproductive investment, growth and mortality rates, egg-size and number, and reproductive life-span became fundamental areas around which theory was developed. Moreover, since these life-history traits were coupled to an organism's rate of increase, these questions were amenable to demographic analyses. One solution was to approach these problems using optimization models, which laid the foundation of classical life-history theory (see for example Sibly and Calow 1986; Roff 1992; Stearns 1992). Optimization models typically define a relationship between traits and fitness, describe trade-offs between traits, and find the combination of traits that maximizes fitness (Stearns 2000). Examples include the physiological approach to the allocation of resources to growth, defense and reproduction adopted by Sibly and Calow (1986), optimization models for age and size at maturity (Stearns and Crandall 1981), optimal reaction norms (Stearns and Koella 1986), clutch size and reproductive

investment (Daan et al. 1990 extending the work of Lack 1947), and life-span and ageing (Reznick et al. 1990). Important departures from these approaches include bet-hedging to spread the "risk" of reproducing (first conceived by Bernoulli 1738; see Real and Ellner 1992) and the habitat template approach (Southwood 1977), both of these being driven by the threat of mortality from abiotic or biotic disturbance. The classical approach led to the claim that life histories are fashioned from an interaction between extrinsic and intrinsic factors. Extrinsic factors are abiotic or biotic ecological selection pressures that influence survival and reproduction, while intrinsic factors are trade-offs among life-history traits that are coupled to lineage-specific constraints on the expression of genetic variation (Stearns 2000).

Classical life-history theory has typically been based on restrictive assumptions, including constant mortality and fecundity rates, often ignoring the phylogenetic constraints that influence the way in which resources are acquired and allocated among species, making it difficult to disentangle extrinsic from intrinsic selection pressures. More recent work has attempted to remove these assumptions and introduce a more realistic ecological framework into which to place the models, including frequency dependence, density dependence, and explicit population dynamics (e.g. Kawecki 1993; Dieckmann 1997; Dieckmann and Doebeli 1999, 2003; Sinervo et al. 2000; Bonsall et al. 2004). These studies were inspired by evolutionary game theory (Maynard Smith 1982; sections 1.5- 1.6). Critically, the development of a more realistic ecological framework has also been associated with a change in the definition of fitness from lifetime reproductive output (RO) or instantaneous rate of increase "r" to frequency dependence and invasion success (a population dynamics version of the Evolutionary Stable Strategy or ESS). Despite these problems, whenever classical theory has been tested in empirical experiments it has often been well supported (Stearns 2000; cf. Daan et al. 1990; Reznick et al. 1990; Stearns et al. 2000). Moreover, an understanding of those factors influencing the survival and reproduction of organisms of different ages or stages, of how traits are

coupled to each other, and of the constraints on the expression of traits, remain important objectives of any investigation of life-history patterns (section 1.7, chapters 2- 5)

1.2. Life-history trait constraints and trade-offs

Central to the evolution and expression of an organism's life-history are the constraints imposed on trait combinations by trade-offs. A trade-off exists when a benefit realized through a change in one fitness trait is linked to a cost incurred through a change in another, "the benefits and costs being calculated in the currency of fitness only" (Stearns, 1992). Trade-offs arise in the allocation of resources to soma, maintenance and reproduction. The timing and prioritization of allocation to these functions gives rise to key life-history trait trade-offs i.e. between large egg size and large numbers, early and late maturation, fast growth and increased reproductive effort. Reproductive effort in this sense is defined as that proportion of the total energy assimilated by an organism that is devoted to reproductive processes, and has traditionally formed a key component of measuring reproductive value in age specific reproductive schedules (Pianka and Parka 1975; see for example Thompson 1984). Without such constraints organisms would essentially be Darwinian demons i.e. individuals that develop and grow rapidly, reproduce continuously, and do not age" (Bonsall et al. 2004).

Numerous studies of a diverse range of taxa in laboratory, semi-natural and natural populations give support to the likelihood of trade-off among traits (e.g. Reznick 1985; Partridge and Sibley 1991; Roff 1992, 2002; Stearns, 1992; Gustafsson et al. 1994; Ots and Horak 1996; Sinervo and DeNardo 1996; Zuk et al. 1996; Roff and Fairbairn 2007). Moreover, the underlying physiological determinant of life history trade-offs has long been a central topic in the context of life-history evolution (Fisher 1930; Tinkle and Hadley 1975; Dunham et al. 1989; Stearns 1992; Roff 1992; Adolph and Porter 1993; Zera et al. 1998; reviews by Townsend and Calow 1981; Bell and Koufopanou 1986; Sibly and Calow 1986; Ricklefs 1991; Sibly and Antonovics 1992; Zera and Harshman

2001). The consensus is that trade-offs are central to the general life history problem (section 1.3) of how, in the presence of reproductive costs, natural selection may result in the evolution of energy allocation patterns that lead to local maxima of individual fitness (Stearns 1992).

Despite the wealth of evidence for trade-offs amongst allocations to life-history traits, how such trade-offs evolve remains largely unanswered from both a theoretical or empirical perspective (Houle, 1991; Chippindale et al. 1996; Reznick et al. 2000; Roff and Fairbairn 2007 and citations therein). Moreover, the term trade-off has sometimes been applied to interactions among traits where there is little or no information as to whether the traits interact functionally (e.g. Zera and Harshman 2001 and citations therein). The term trade-off, in the context of physiological life-history studies, should represent a hypothesis concerning the cause of a negative trait association. If this association cannot be categorically proven then the term potential trade-off is preferable (Roff and Fairbairn 2007). Empirical approaches to identify life history trade-offs and their physiological causes may be grouped into three categories: i) measurement of phenotypic correlations on unmanipulated individuals, ii) genetic analyses, and iii) experimental manipulation of phenotypes. The relative importance of each approach has been extensively debated (see for example Zera and Harshman 2001 and citations therein). To understand the evolution of life-history trade-offs all three approaches should be integrated. As a first step, phenotypic correlations derived from unmanipulated individuals in the field or laboratory are useful because they can detect trade-offs and the potential physiological causes of trade-offs (review by Zera and Harshman 2001 and citations therein; chapter 3).

1.3. The general life-history problem

The optimal allocation of resources to growth and reproduction throughout an organism's life is known as the general life-history problem, which has traditionally been approached with general allocation models (Stearns 2000 and citations therein). Central to these models are estimates of age-specific reproductive investment, growth and survival. From these models predictions of the optimal age at maturity or life-span, for example, may be derived and compared with empirical data (see Heino and Kaitala 1997a,b for an example of optimal allocation in clams). This approach has yielded some of the central predictions of life-history theory (Michod 1979; Roff 1981; Mangel and Clark 1988; Reznick et al. 1990; Kozlowski 1991, 1992; Real and Ellner 1992; Charlesworth 1994; Heino and Kaitala 1999). All of which depend on the assumption that the cost of reproduction is paid in future mortality or future reproduction or both. A potential disadvantage of the general life-history paradigm or problem is its application to complex life-histories in indeterminately growing organisms such as marine benthic invertebrates with both larval and adult phases (Strathmann 1993; Giangrande et al. 1994). One solution has been to address the different trade-offs experienced by each stage separately such that the life-history is represented as an active decoupling between specialization's at different stages (Moran 1994). For example, the pelagic larval stage in marine invertebrates could represent an adaptation to dispersal and habitat selection, the adult stage specialization for reproduction and growth (Wray and Raff 1991; Pechenik 1999). Another interpretation is that complex life cycles may be viewed as an "adaptive size-specific shift in the ecological niche by means of metamorphosis" (Wilbur 1980). For example, at small sizes planktonic larvae may be more advantageous than a benthic form. Thus size at metamorphosis is a key trait in species with complex life cycles.

Despite the debate over the appropriate approach, a wide variety of models have been proposed to define the optimal allocation of resources to growth and reproduction. Essentially, simple models in a constant environment emphasize that organisms should

adopt a "bang- bang" option of setting reproduction to 0 until growth ceases, after which all energy is allocated to reproduction during one (semelparity) or repeated (iteroparity) reproductive events (reviews by Kozlowski 1992; Perrin and Sibly 1993). This strategy, known as determinate growth, has been demonstrated for many vertebrates (Sebens 1987), yet many organisms exhibit indeterminate growth and iteroparity where allocation to growth and reproduction may occur throughout the organisms life e.g. in perennial plants, mollusks, echinoderms and fish (Heino and Kaitala 1999). Together, these forms of growth help to explain the enormous variation in patterns of growth among organisms (Sebens 1987). Animals with indeterminate growth must trade-off reproduction against growth throughout their lives (Stearns 1992; review by Heino and Kaitala 1999). Allocation to reproduction within a given season in indeterminately growing organisms has a profound influence on future size and fecundity. Body size affects not only fecundity but also many ecological factors, including inter- and intraspecific interactions, and susceptibility of prey to predation (see for example Heino and Kaitala 1999; section 1.5, chapter 3). Moreover, the lifetime resource allocation strategy is further complicated by environmental factors such as changes in the availability of resources and the risk of dying (sections 1.4, 1.7, chapter 5).

1.4. Plasticity of allocation

The traits involved in resource allocation among maintenance, growth and reproduction may also show phenotypic plasticity, i.e. the phenotypic expression of the traits depends on the environment. Several studies have addressed the question of the control of resource allocation at the whole-organism level. For example, in many laboratory and field experiments on bivalves, cladocerans, insects, and mammals, allocation to maintenance or storage took precedence over allocation to reproduction under nutrient-poor or stressful conditions (Bayne et al. 1983; MacDonald and Thompson 1985; Perrin et al. 1990; Boggs and Ross 1993; Jokela and Mutikainen 1995; Rogowitz 1996). Although if conditions are stressful enough and death is likely, investment to

reproduction at the expense of survival has been documented (Thompson 1983; Boggs 1992). More recently, Ernande et al. (2004) have emphasized the need to treat organisms as integrated entities characterized by numerous correlated traits e.g. growth, maintenance and reproduction. Generally, it is unlikely that different traits co-vary plastically such that they all influence fitness in the same direction (Boudry et al. 2002; Ernande et al. 2004). Indeed, any plastic increase of resource allocation in one trait should be correlated with a decrease in the others (Ernande et al. 2004; chapter 3).

The options available to an organism in allocating resources among maintenance, growth and reproduction are constrained by its life history. Short-lived species by definition have less time to reproduce than long-lived animals and thus fewer opportunities to delay reproduction (Stoeckmann and Garton 2001). Hence short-lived species increase fitness through earlier reproduction and vice-versa for long-lived species (Pianka and Parka 1975; Stoeckmann and Garton 2001). Allocation priorities are different in the first years of life in short and long-lived bivalves (for example, freshwater zebra mussels *Dreissena polymorpha* <4 years old, and pearl mussels *Margaritifera margaritifera* >100 years old (Bauer 1998; Stoeckmann and Garton 2001)), which in turn influences their allocation patterns in variable environments and response to degree of environmental stress (Macdonald and Thompson 1985; Rodhouse et al.1985; Macdonald and Bayne 1993; Stoeckmann and Garton 2001) and/or their terminal body-size. If, however, there is a risk of immediate mortality for either long-lived or short-lived species, the short-term response, as summarized in Boggs 1992, should be to increase reproduction and die (Bayne et al. 1983; Thompson 1983; Reimer and Tedengren 1999; Stoeckmann and Garton 2001). As the value of "survival" decreases with age (Williams 1966; Pianka and Parka 1975), theoretical models on optimal energy allocation also predict an increase in reproductive allocation or effort with age, which is correlated with size in indeterminately growing organisms. In bivalves, with the exception of extremely long lived freshwater pearl mussels (Bauer 1998), this trend is common in both short and

long-lived species (Vahl 1981; Bayne and Newell 1983; Thompson 1984; MacDonald and Thompson 1985; Rodhouse et al. 1985).

The examples above typically relate to cases where limited resources are allocated to growth, reproduction and survival. Sibly and Calow (1986) emphasized that for a more complete representation of how an organism's life-history is adapted to its environment, studies of resource allocation should be further subdivided into growth, defense, reproduction and maintenance. Allocation to growth to reach a size refuge from predation is possible (see for example Paine 1976; Seed and Brown 1978), although there are other ways to increase an organism's resistance to predation (chapter 4). Examples in *Mytilus* species include allocation to thicker shells and increased attachment of *Mytilus* species in response to the risk of detachment and breakage by crabs (Leonard et al. 1999; chapter 4). Overall, predators may be a key cause of mortality in organisms that do not experience extreme episodes of indiscriminate disturbance and therefore represent a critical selection pressure driving the allocation strategy.

Rather than allocate resources to costly defenses throughout an organism's life, where predators are patchy in space and time "costly" defenses may be induced only in response to an immediate threat of predation (Havel 1987; chapter 4). Predator-induced defenses have been demonstrated in many organisms: algae, vascular plants, protozoans, cnidarians, cladocera, gastropods, amphibians, fishes and birds (Leonard et al. 1999 and citations therein). Invasive predators may also precipitate evolutionary change in the communities that they penetrate. Freeman and Byers (2006), for example, suggested that there has been rapid evolution of an inducible morphological response in blue mussel spp. to *Hemigrapsus sanguineus* within 15 years of its introduction to Southern New England (USA) (but see Rawson et al. 2007). Nevertheless, these studies of inducible defenses do not usually quantify allocation to growth, reproduction and maintenance, making it difficult to empirically or theoretically determine the adaptive significance of inducible defenses in terms of the population dynamics and invasion fitness of the prey

species. For example, an alternative approach to the threat of instantaneous predation is simply to invest in reproduction and die (Boggs 1992). Here the reproductive contribution of an individual is transferred to the next generation, thereby increasing fitness rather than resisting disturbance (Southwood 1977; section 1.2, chapter 3).

1.5. Future directions for life-history theory- species diversity and diversification

Recently, the application of life-history theory has been extended to interactions among, or evolution of, ecologically distinct phenotypes or species. Life-history trait trade-offs that influence ecological interactions among or within species may promote the coexistence of divergent phenotypes within or among species or the emergence of new species in sympatry through evolutionary branching (Diekmann 1997; Diekmann and Doebeli 1999, 2000; Bonsall et al. 2004; Doebeli and Diekmann 2005). Resource or apparent competition may be a major driving force for sympatric speciation (Doebeli and Diekmann 2000) and could explain, for example, the large variation in body size among stickleback species (Nagel and Schluter 1998; Rundle and Schluter 1998). Moreover, the prevailing disturbance regime is also a key component of co-existence among species, particularly in sessile plants and animals (Petraitis et al. 1989; section 1.6, chapter 3). This is because life-history differences differentiate species through their responses to disturbance, thereby helping to define the spatial- temporal niches required for co-existence (Shea et al. 2004). "Knowledge of the life-history strategies of the component species is therefore critical for an ecological interpretation of the co-existence mechanism" (Roxburgh et al 2004; sections 1.6- 1.7).

In summary, key life-history traits arise from the allocation of limited resources to maintenance (which affects survival), growth (which influences age/size at maturity and fecundity) and reproduction (which influences dispersal and colonization abilities) (Fisher 1930; Stearns 1992; Charlesworth 1994; Tilman 1994, 2000; Roff & Derose 2001; Ernande et al. 2003). To date, few studies have directly compared resource

allocation in species co-existing in variable environments (section 1.3, chapter 3). Indeed, such comparisons may provide insight into the nature of reproductive costs and the plasticity of allocation that allows different life-history strategies to co-exist in fluctuating environments. The role of life history trait trade-offs pertaining to the co-existence of interacting species, which forms the motivation for this thesis, is outlined in more detail below (section 1.6). These theories have not yet been extended either to model or to collect empirical life-history data to assess the establishment success of hybrids produced by closely related interbreeding species such as those in the *Mytilus* complex (chapter 3). Such investigations would facilitate a more integrated understanding of species diversity and diversification.

1.6. The application of the general life-history problem to the co-existence of closely related sibling species

Biodiversity is likely maintained by a continuum of neutral (stochastic) and niche (deterministic) processes (Bonsall et al. 2004; Tilman 2004; Gravel et al. 2006; Adler et al. 2007; Clark et al. 2007; Nekola and Brown 2007). Finding the relative contribution of each process for a given species guild is a major challenge (Adler et al. 2007), although recent advances in statistical applications e.g. derivations of invasion (Diekmann 1997; Diekmann and Doebeli 1999; Doebeli and Dieckmann 2000; Bonsall et al. 2004; Bonsall and Mangel 2004; Mangel et al. 2007; Schoolmaster and Snyder 2007) or Baysian analysis (Clark 2003; Clark et al. 2003; Clark 2005; Clark et al. 2007) that require an understanding of the role of inter-individual and inter-specific life-history differentiation and demographic or environmental stochasticity in temporal and spatial dimensions offer some potential (see chapter 3). Generally, greater ecological differentiation within guilds of species should enhance biodiversity (Dieckmann and Doebeli 2000; Tilman 2004; Harpole and Tilman 2007). Where species do not clearly differentiate along resource or physiological gradients, divergent allocation to life-history traits that influence their relative ecological interactions, in addition to stochastic processes, may help to explain

the puzzling abundance of closely related or "sibling species" competing for space and resources (Huisman et al. 2001; Calcagno 2006).

The discovery of morphologically similar sibling marine species (Knowlton 1993) that may readily interbreed and occupy a similar resource niche has provided a unique opportunity to identify the role of interacting sibling species' life-history patterns in maintaining high diversity within species guilds. This approach requires an understanding of each species' life-history, particularly the "cause and effect" of evolutionary or ecologically important traits. To address "cause and effect" researchers identify selection pressures (cause), choose a life-history problem and explain trait divergence (effect) while maintaining awareness of phylogenetically determined developmental constraints (Stearns 2000). Selection pressures common to closely related organisms include density, resource availability, predation, competition for space or food, and the likelihood of inter-breeding, while key life-history trait problems include age and size at maturity, egg-size and number, and longevity (Stearns 1992; Roff 1992; Charlesworth 1994).

For organisms with complex life-histories, e.g. marine invertebrates with distinct larval and adult phases, investigations risk fragmentation as models are designed to address specific life-history problems without consideration of the effect of one life-stage on the other (Stearns 1992). In particular, the general life-history problem, which concerns the optimal allocation of resources to growth, survival and reproduction (Shaffer 1983), drives a more unified approach to understanding an organism's life-history and associated ecological differentiation (section 1.3). Furthermore, the general life-history problem is amenable to mathematical modeling. For example, estimates of survival (lx) and fecundity (mx) schedules provide the foundation for describing the population dynamics of interacting species (Dublin and Lotka 1925). Derivations of this problem are playing an increasingly important role in understanding species biodiversity, for example in non-equilibrium approaches incorporating ecological differentiation, particularly adaptive dynamics and invasion likelihood (Diekmann and Doebeli 1997,

1999, 2000, 2003; Bonsall et al. 2004) or recent hierarchical Baysian approaches (Clark et al. 2007).

The current thesis addresses the cause and effect of allocation patterns in co-existing, hybridizing, sibling blue mussels *Mytilus edulis* and *M. trossulus* competing for space and other resources. More specifically, it deals with the effect of resource availability on resource allocation to growth (soma), survival and reproduction and how the resultant ecological differentiation may both equalize and stabilize (Chesson 2000) co-existence between the species (chapter 3). To date, ecological factors influencing the distribution and abundance of sibling species within the *Mytilus* complex, for example, are not conclusive. There is some evidence for physiological differentiation along temperature or salinity gradients (Qiu et al. 2002; Braby and Somero 2006) and differentiation according to wave-exposure (Gilg and Hilbish 2000; but see Hilbish et al. 2002), but there is still considerable scope for distributional overlap in the natural environment. This is especially true of *Mytilus edulis* and *M. trossulus* co-existing in Atlantic Canada where the two species do not differentiate along any observed temperature or salinity gradients (Gardner and Thompson 2001; Moreau et al. 2005). Moreover, the observation that *M. trossulus* is sometimes relatively more abundant in the upper intertidal (Bates and Innes 1995; Miranda 2004; Braby and Somero 2006), which is subject to more extreme temperatures in the summer, is inconsistent with the finding that *M. trossulus* is less tolerant of high temperature in the laboratory (Miranda 2004). Since key invertebrate mussel predators are more abundant in the lower shore and less tolerant of salinity (Seed and Suchanek 1992), other factors, including differential susceptibility to predation and the ability to induce defenses, require examination and may complicate the interpretation of the data (section 1.5; chapter 4).

Closely related *Mytilus* species competing for space and other resources are also more likely to inter-breed because of spatial overlap facilitated by their planktonic larvae (Bates and Innes 1995; Suchanek et al. 1997; Bierne et al. 2002, 2003). In this context

divergent life-histories may contribute to other pre- or post-zygotic barriers to hybridization (Toro 1999; Miranda 2004; Bierne 2006; Liu 2007; Springer and Crespi 2007), for example by establishing successional dynamics or spatial segregation that concentrate intraspecific rather than interspecific interactions (Chesson 2000), or by erecting phenotypic barriers to hybridization (e.g. egg size (Levitan 2006)). Moreover, the viability of hybrids and the likelihood of "invasion success" (Dieckmann 1997; Dieckmann and Doebeli 1999, 2000, 2003) may be correlated with their life-history and resultant ecological differentiation compared with the parental species. In other words, how reproductively viable are the hybrids compared with the parental species?

Previous studies of *M. edulis* and *M. trossulus* in sympatry have shown little inter-specific variation in larval and juvenile viability (growth and survival) in the laboratory, despite large differences in egg size (Toro et al. 2002; Miranda 2004; Liu 2007), a higher proportion of *M. trossulus* generally being observed at larval and post-settlement juvenile stages (Toro et al. 2004; Miranda 2004; Stapleton 2007; Wang J., *pers. comm.* 2007). The frequency of *M. trossulus* drops considerably in relation to *Mytilus edulis* in size classes of ~30- 60mm (Toro et al. 2004; Miranda 2004), indicating a critical period of divergence in the species' life-history. Given the general life-history problem of allocating limited resources to reproduction which trades off with survival and/ or growth, this divergence is hypothesized (in the current thesis) to arise from greater allocation earlier in life to reproductive rather than somatic or maintenance functions in *M. trossulus* than in *M. edulis*.

Following quantification of the reproductive cycle and reproductive output (chapter 2), a key objective was to determine the plasticity of survival, somatic and reproductive traits in *M. edulis, M. trossulus* and their respective F1 hybrids (section 1.7). I specifically focus on how the three traits respond to variability in site productivity within each genotype and the degree to which each trait differs among the genotypes (chapter 3, section 1.7). This work was complemented by measurements of ecologically

important morphological or somatic traits (chapter 3, section 1.7). The findings determined the design of the next series of experiments, which focussed on describing predator inducible defenses or density dependent intra- and inter-specific interactions of *M. edulis* relative to *M. trossulus* (chapters 4, 5, section 1.7). These experiments aimed to both explain the constraints placed on each species and their F1 hybrids by their allocation patterns and to describe key ecological interactions that may help to explain the distribution and abundance of *M. trossulus* and *M. edulis*, as well as their potential to coexist sympatrically (see Chapter 6).

1.7. Rationale and objectives

1.7.1. Rationale

Where closely related species (e.g. *M. trossulus* and *M. edulis*) do not clearly differentiate along resource or physiological gradients, resource allocation to traits that enhance their ecological differentiation in fluctuating environments could stabilize their co-existence (sections 1.5, 1.6). Accordingly, a key objective was to describe the species allocation strategies and associated ecological differentiation (objectives 1 and 2 below). The findings determined the design of a subsequent series of experiments, which focussed on establishing the species relative susceptibility to biotic disturbance and how their allocation strategies might mediate the effects of such disturbance (section 1.5; objectives 3 and 4 below). The findings were then synthesized in a summary chapter to discuss the species distribution, abundance, and potential to co-exist and interbreed under varying disturbance regimes (chapter 6).

1.7.2. Objectives (see also section 1.7.1)

1. To describe the gametogenic and body condition cycles of *M. edulis*, *M. trossulus* and their F1 hybrids. This information was used to estimate reproductive output and reproductive effort (objective 2, chapter 3).

2. To compare survival and allocation of resources to somatic growth and reproduction in *M. edulis*, *M. trossulus* and their F1 hybrids from sites with different productivity's (chapter 3).

3). To compare *M. edulis* and *M. trossulus* predator induced defensive allocation and associated susceptibility to predation (Chapter 4). A secondary aim was also to determine whether or not the observed differences between *M. edulis* and *M. trossulus* in adductor muscle mass and shell thickness were attributable to predator inducible plasticity (chapter 4).

4. To compare how *M. edulis*, *M. trossulus* and interspecific mixtures of *M. edulis* and *M. trossulus* respond to crowding in terms of their growth, survival and shell morphology (chapter 5).

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Chapter 2

Reproductive trends in *Mytilus edulis, Mytilus trossulus* and their F1 hybrids.

2.1. Introduction

The discovery of sympatric sibling *Mytilus* species has led to a considerable effort to understand their distribution and potential to co-exist and hybridize (Gosling 1992; Gardner 1994ab; Hilbish et al. 1994, 2000, 2002; Rawson and Hilbish, 1995a,b; Saavedra et al. 1996; Suchanek et al. 1997; Comesaña et al. 1999; Penney and Hart 1999; Toro et al. 2002, 2004; Gilg and Hilbish 2003). *Mytilus* species are highly fecund broadcast spawners that develop a dispersing larval stage before settlement, which in turn facilitates spatial overlap. Moreover, because of the production of viable hybrids, explanations of the distribution and abundance of sibling *Mytilus* species are focussed on two fronts- first, the co-existence of each species, and second, mechanisms by which interbreeding and the potential loss of species identity with extensive hybridization are prevented (Beaumont et al.1993; Beaumont and Abdul-Matin 1994; Bates and Innes 1995; Gardner and Thompson 2001; Secor and Hilbish 2001; Bierne et al. 2002; Toro et al. 2002, 2004; Gilg and Hilbish 2003; Rawson et al.2003; Miranda 2004; Liu 2007). To date, the potential of *Mytilus* species to co-exist in the former context is not well understood, and most investigations have focussed on environmental rather than life-history attributes to explain the distribution of the species (Qiu et al. 2002; Gilg and Hilbish 2003; Miranda 2004; Moreau et al. 2005; Braby and Somero 2006).

The *Mytilus edulis- trossulus* hybrid zone in the northwest Atlantic, in particular, is poorly understood in terms of the potential of *M. edulis* to co-exist with *M. trossulus*. For example, where *M. edulis* and *M. trossulus* co-occur in Newfoundland there is limited evidence for clear zonation along temperature or salinity gradients or according to height in the intertidal zone (Bates and Innes 1995; Gardner and Thompson 2001; Miranda 2004), although *M. trossulus* generally decreases in frequency with increasing

size class when co-existing with *M. edulis* (Comesaña et al. 1999; Toro et al. 2004). This may possibly be explained by greater reproductive allocation in *M. trossulus* than in *M. edulis*, resulting in reduced growth or survival in the former, because greater reproductive allocation earlier in life enhances rates of increase, generally at the expense of growth and survival (Dublin and Lotka 1925; Sibly and Calow 1986; Stearns 1992; Roff 2001). Moreover, where closely related or sibling species with different reproductive strategies compete for space and other shared resources, trade-offs between reproduction, growth, and survival lead to ecological differentiation that increases the likelihood of co-existence (Chesson 2000; Calcagno et al 2006; chapters 3-6).

Before any estimates of reproductive allocation can be made in *Mytilus* species, the reproductive cycle must be understood, specifically the period of gametogenesis and spawning. *M. edulis* and *M. trossulus* in Newfoundland demonstrate an opportunistic reproductive strategy, where gametogenesis does not proceed over winter but progresses rapidly through spring and early summer, with spawning taking place between June- July (Thompson 1984b; Toro et al 2002). Gametogenesis occurs mainly in the mantle tissue, but reproductive tissue can also be found in the visceral mass and mesosoma (Bayne et al. 1978; Lowe et al. 1982; Newell et al. 1982). The most reliable and detailed information regarding the reproductive cycle is that obtained from histological preparations of mussels sampled at bi-weekly to monthly intervals (Seed and Suchanek 1992). Such measurements can usefully be augmented by gonado-somatic or body-condition indices (Toro et al. 2002). Reproductive output can be approximated in mussels with gonads that are ripe, highly developed or have just initiated spawning (Seed and Suchanek 1992; Toro et al. 2002), and can be measured by direct "inducement" to spawn, or by estimates of gonad weight derived from the gamete volume fraction (Lowe 1982) multiplied by the weight of dried reproductive tissues (mantle + mesosoma), or simply the dried mantle + mesosoma weight. Since approximately 90% of the mantle is composed of reproductive tissue, the latter is a reliable estimate of "potential" reproductive output, provided that the

gonads are highly developed, ripe, or have just initiated spawning (Seed and Suchanek 1992).

To better understand the potential of *M. edulis* and *M. trossulus* to co-exist in Newfoundland the main objective of this study was to define their reproductive cycles in order to determine whether *M. trossulus* exhibits greater reproductive output earlier in life than *M. edulis*. Moreover, by contrasting the reproductive cycles of hybridizing *Mytilus* species the potential for reproductive isolation through asynchronous spawning cycles could be determined (Toro et al. 2002). Finally, although pre-and post-zygotic barriers to hybridization have been identified, little is known about the reproductive viability of first generation (F1) hybrids in the wild (Miranda 2004; Liu 2007). The reproductive cycle and reproductive output of F1 hybrids were also compared with those of the parental species. By following laboratory produced cohorts of each species and their F1 hybrids it was possible to control for the effects of age (Bayne et al. 1983; Kautsky 1982; Sprung 1983; Thompson 1984a; Rodhouse et al.1985) and position (Okamura 1986), both of which are potentially confounding factors in reproductive studies. Furthermore, information on the reproductive cycle obtained in this study was used to estimate reproductive effort and resource allocation in a companion study of the two species and their F1 hybrids (chapter 3).

2.2. Materials and methods

2.2.1 Laboratory production of mussels

Mussels (*M. edulis* and *M. trossulus*) were obtained from Bellevue (NL) and identified with two allozymes (MPI and EST-D), 4 nuclear DNA markers (ME, ITS, MAL and PLIIa) and one mitochondrial DNA marker (COIII) (Miranda 2004). Pure-species families (5 families per species) were then produced in the laboratory in July 2000. In addition, F1 hybrid families (5 families for each of the two reciprocal hybrid crosses) were produced from *M. edulis* females crossed with *M. trossulus* males (hybrid *edulis*) and *M. trossulus* females crossed with *M. edulis* males (hybrid *trossulus*). Pure species families and hybrid families were collectively referred to as "genotypes". Prior to deployment, all families within each genotype were pooled and reared in identical conditions for 3 years (Miranda 2004). Mussels were initially reared for 16 months in the laboratory (July 2000-November 2001), where they were fed a standard hatchery rearing diet consisting of mixtures of live algae and raw seawater in a partial recirculation system.

2.2.2. Deployment, sampling and dissection of mussels (cohorts 3 and 4 years old)

Two cohorts were used in the current experiment. Mussels from the first cohort (3 years old) were transferred from the laboratory (section 2.2.1) in November 2001 to a mussel farm in Notre Dame Bay for 12 months, after which they were retrieved in November 2002, returned to the laboratory, and reared in the laboratory as described above (section 2.2.1). At age 3 years these mussels were deployed to Long Harbour (Placentia Bay) and Trinity (Trinity Bay) in May 2003. The second cohort (4 years old) was also obtained from the same pool of laboratory produced mussels (section 2.2.1) but was deployed in November 2001 to Long Harbour as part of another growth experiment (Miranda 2004) where the mussels remained until December 2003. The second cohort

(age 4 at the time of this experiment) was then returned to the laboratory in December 2003, reared as described in section 2.2.1 and deployed to Long Habour and Trinity in December 2003. The second cohort was then returned to the laboratory for several days in late May 2004 from Trinity, or mid June 2004 from Long Harbour, for marking (see below) and returned to the two sites.

To introduce a growth check, each individual mussel was marked 10mm back from the posterior shell margin with a Dremmel Tool™ before deployment. During June 2003 and December 2003 cohorts of each genotype (N ~200 and 100 mussels respectively) were randomly assigned to 8 pearl nets (2 replicates per genotype) anchored off-bottom at Long Harbour (Placentia Bay) and Trinity (Trinity Bay), Newfoundland. To prevent position effects these nets were re-arranged at each sampling time. From June-December 2003, monthly samples were taken of 20-30 mussels per genotype per site from the first cohort (age 3). For the second cohort monthly samples of 20-30 mussels per genotype were taken from each site between late May (Trinity) or mid June (Long Harbour) and December 2004. Due to high cumulative mortality over their life-time, *M. trossulus* and hybrids were not available for sampling after August 2004 at Long Harbour, while hybrids were not available for sampling at Trinity after August 2004. For histological examination (section 2.2.3), a piece of mantle tissue ~0.20cm^2 in size was dissected from the mantle, weighed, fixed in Davidson's AFA fixative for 48 hours and transferred to 70% ethanol. Because reproductive tissues in *Mytilus* species are homogeneously distributed throughout the mantle (Lowe et al. 1982) only one piece of mantle tissue was preserved from each mussel. Mussels were subsequently frozen for later dissection, when the remaining gonadal tissue in the mantle, the rest of the soft tissues and the shell were removed, weighed separately and dried to constant weight for at least 72 hours. Dry weights of the mantle and remaining somatic tissue were determined and shell morphometric measurements made (chapter 3). Total dry weights of mantle and body tissue corrected for tissues removed for histology were calculated using weight loss in dried tissue as an indicator of water content in tissues that were not dried.

2.2.3. Histological assessment

Samples of mantle tissue stored in 70% ethanol were dehydrated in a graded series of ethyl alcohol (50, 70, 80, 95 and 100%), cleared in Hemo-De and embedded in paraffin wax. Sections 7μm thick were prepared and stained with hemotoxylin and eosin. Representative samples of horizontal sections through the mantle tissue were cut for each individual, the section with the largest area being chosen to assess reproductive state. To validate gonado-somatic index (GSI) estimates (see below), quantify the influence of sex on gamete output, and compare sex ratios, samples were processed throughout each six-month sampling period. Due to logistical and time constraints only a small number of samples relative to the number of mussels sampled for dry weight analyses could be processed i.e. ~250 individuals of the 1700 mussels sampled during 2003 and 2004. Reproductive condition of the two types of F1 hybrids were almost identical to that of the species used as the female parent. Preliminary examination of histological slides had shown that gamete development in both species was greatest at peak GSI, when both males and females were either ripe or had begun spawning.

To provide a more detailed assessment of reproductive state at peak GSI (Lowe et al. 1982), the gamete volume fraction (GVF) of *M. edulis* and *M. trossulus* was calculated with imaging software (Image J) using high-resolution digital images of each section taken at 100x magnification. Three fields per section were randomly chosen for calculation and the average percent coverage by stained gametic tissue was calculated as the gamete volume fraction (GVF). In a small number of cases, where tissue was thin or fragmented, percentage cover was estimated visually. To test the reliability of this visual method, GVF estimates were also made from sections that were not fragmented; the error associated with visual assessment was estimated at ± 2.5%. Correction factors were also applied to data for sperm and eggs to compensate for overestimates resulting from particle size (Secor et. al 2001; Lowe, D.M. *pers .comm.* After arcsine-square root transformation to achieve normality, GVF values at peak gamete ripeness were analyzed

for each site in 2003 or 2004 by a Two-Way ANOVA with genotype and sex as fixed factors (Type III SS, R Project for Statistical Computing). To reduce the chance of Type I errors, significance levels for all parameters used in the GVF ANOVAs were adjusted with the Bonferroni method (Type III SS) (Sokal and Rohlf 1995). To test the hypothesis that males and females occurred at a 1:1 ratio, sex ratios were determined for each genotype x site combination and compared by G-tests (Pop Tools in Microsoft Excel™). A small number of sections (<10) were excluded from the analysis due to extensive fragmentation.

2.2.4 Gonado-somatic index (GSI)

At each sampling date a gonado-somatic index (GSI: dried mantle weight / total weight of dried soft tissues) was calculated and expressed as a percentage. The accumulation and release of gonadal material and the utilization of stored energy products during winter determine the temporal pattern of the GSI (Toro et al. 2002). Due to logistical constraints and unpredictable weather in the fall of each year, it was not possible to sample at exactly the same dates at each site. Consequently a multivariate analysis including all measured variables from the same individuals was not possible (see also chapter 3.2.3). As such, to reduce the probability of Type I error when selecting samples from the same data-set, significance levels were Bonferroni corrected (Sokal and Rohlf 1995). Two-Way ANOVAs of arcsine-square root transformed GSI data were carried out for each year class and site separately, with genotype and date as fixed effects and variation among pearl nets as a random effect (Type III SS, R Project for Statistical Computing). Net effects were subsequently dropped from the analyses because they were not significant. Where significant interactions between genotype and date were evident, GSI values between genotypes within each site were contrasted at peak GSI. Peak GSI was determined for each genotype following pair-wise contrasts among dates within each site. Where GSI peaked over more than one date, contrasts of peak GSI among genotypes were made using dates where peak GSI corresponded with the greatest gamete

development (see histological analyses 2.2.3). For graphical representation of the findings arcsine back-transformed means and adjusted confidence intervals are presented (figures 2.1- 2.2). Confidence intervals were adjusted with the Bonferroni method in accordance with the pair-wise contrasts mentioned above.

2.2.5. Gamete investment and egg-size

Gonad weight, a measure of potential reproductive output or gamete investment, was estimated from the dried mantle in ripe mussels or mussels that had just begun to spawn. Since >90% of the mantle was composed of reproductive tissues, mantle weight was a reliable indicator of gonad weight and hence reproductive output. For each site in 2003 or 2004, genotype differences in potential reproductive output were compared by ANCOVA with genotype as a fixed effect and shell surface area as a co-variate (to adjust for differences in body-size). The surface area of the shell was approximated using the formula:

$$A = l(h^2+w^2)^{0.5}0.5\pi \qquad \text{eqn 1}$$

Where A is surface area (cm^2), l is length (cm), w is width (cm) and h is height (cm) (Beadman et al. 2003). Because shell surface area is strongly correlated with shell volume, A is a reliable measure of body size (Freeman and Byers 2006). To reduce the probability of Type I error when selecting samples from the same data-set, significance levels for all parameters used in the ANCOVAs (Type III SS, R Project for Statistical Computing) were computed with the Bonferroni method (Sokal and Rohlf 1995). Identical ANCOVAs using initial shell length or soft tissue weight as a covariate produced similar results (see appendix section 7.1 for estimation of initial shell length or soft tissue weight). For all ANCOVA contrasts slopes were homogenous. Pair-wise contrasts between genotypes were computed with the Bonferroni method (Type III SS). To determine egg size for each female, thirty oocytes with a nucleolus (10 from each histological section) were measured across the longest axis using imaging software

(Image J). An ANOVA of egg diameter (averaged for each individual following the measurement of 10 eggs for each histological section) was carried out for each year and site separately, with genotype as the fixed factor (Type III SS, R Project for Statistical Computing). Where significant genotype effects were evident, pair-wise comparisons were adjusted with the Bonferroni method (Day and Quinn 1989).

2.3. Results

2.3.1. Histological analyses

Examination of histological sections confirmed that the follicles of both species and all hybrids contained ripe or almost ripe eggs and sperm in late July 2003 at Trinity and Long Harbour and late May and mid-June 2004 at both sites. In all cases these dates corresponded with peak GSI (Figures 2.1- 2.2). Following the decline in GSI, all mussels were either undifferentiated or resorbing gametes, confirming that spawning was complete (Figures 2.1- 2.2). An ANOVA of GVF at each site during 2003 or 2004, with genotype and sex as fixed factors, lead to two important results. Firstly, because sex did not explain a significant amount of the variation in GVF values at peak GSI in either 2003 or 2004 ($F_{1,47} < 2.5$, $P > 0.05$ at each site), sexes were pooled for the analyses of peak GSI and fecundity. Secondly GVF values at peak GSI were not significantly different between species ($F_{1,47} < 1.6$, $P > 0.05$ at all sites during 2003 or 2004) thereby confirming that both species had attained a similar developmental state at peak GSI. Sex ratios (at peak GSI) did not differ significantly from a 1:1 ratio (G-test $P<0.05$) for *M. edulis* (E) or *M. trossulus* (T) deployed at Trinity during 2003 (E♀1.00: E♂1.00, N= 28; T♀0.96: T♂1.04, N= 23) or 2004 (T♀1.17: T♂0.83, N= 26; E♀0.77: E♂1.23, N= 24) and Long Harbour during 2003 (E♀1.08: E♂0.92, N= 24; T♀1.11: T♂0.89, N= 27) or 2004 (E♀0.96: E♂1.04, N= 25; T♀1.33: T♂0.37, N= 24).

2.3.2. Gonado-somatic index (GSI) cycle

In 2003, the GSI of all genotypes increased to a peak from June to August at Long Harbour and Trinity as the mussels were preparing to spawn or had begun spawning (Figure 2.1). Declines in GSI from peak values were evident in *M. edulis* and *M. trossulus* at Long Harbour and *M. trossulus* at Trinity in 2003. In 2004, as the mussels were preparing to spawn or had begun spawning, GSI increased to peak at Trinity around May- June in all genotypes, while GSI peaked during June at Long Harbour in all genotypes (Figure 2.2). Moreover, in 2004 (in contrast to 2003), GSI dropped rapidly in all genotypes (Figure 2.2). Peak GSI was significantly greater in *M. trossulus* than *M. edulis* in both 2003 and 2004 at both sites (albeit much less pronounced in 2004), while the decrease in GSI following spawning was more marked at both sites in 3 or 4 year old *M. trossulus* than in *M. edulis* of the same age (Figures 2.1-2.2).

Despite large significant inter-specific differences in peak GSI, GVF was not significantly different between *M. edulis* and *M. trossulus*, which was confirmed by visual assessment of mantle cross sections (see 2.3.1). The magnitude of the difference in peak GSI between genotypes reflected a greater proportion of soft tissues by weight devoted to the mantle in the *M. trossulus* mussels sampled compared with the other genotypes. Peak GSI values for both hybrid groups, except hybrid *trossulus* sampled from Trinity in 2004, were significantly lower than for *M. trossulus* at both sites in both years but not significantly different from *M. edulis* at both sites in both years. Moreover, peak GSI was not significantly different between hybrid genotypes at both sites during 2003 or 2004. From histological sections it was clear that each F1 hybrid group and *M. edulis* were spawned out by December at both sites in 2003 (section 2.3.1). The lack of a clear decline in GSI values, for the hybrid groups at both sites and *M. edulis* at Trinity in 2003, likely reflected an increase in storage tissues accumulated in the mantle relative to other somatic soft body tissues during and after spawning. This reveals a deficiency in using the GSI cycle for estimating the period of spawning in younger mussels and emphasizes the need for an approach that integrates histological methods.

2.3.3. Gamete investment (size corrected gonad weight) and egg-size

M. trossulus had a significantly greater gonad weight (corrected for body-size) than *M. edulis* at Long Harbour in 2003 and at Trinity in 2003 and 2004 (Figure 2.3, Table 2.1). In contrast, the size corrected gonad weight of *M. trossulus* was not significantly different from that of *M. edulis* at Long Harbour in 2004. At Trinity in 2003 and 2004, size corrected gonad weight did not differ between hybrid groups nor between each hybrid group and its female parent. Moreover, at Long Harbour in 2003 and 2004, the size corrected gonad weight of hybrid *trossulus* was significantly lower than that of *M. trossulus*, while that of hybrid *edulis* was not significantly different from that of *M. edulis*. There was no significant difference between the size corrected gonad weight of *M. edulis* and that of either hybrid group.

M. trossulus developed significantly smaller eggs than *M. edulis* at both sites during both 2003 (Long Harbour $F_{1,21}=212.5$, $p<0.001$; Trinity $F_{1,21}=15.7$, $p<0.01$) and 2004 (Long Harbour $F_{1,20}=77.6$, $p<0.001$; Trinity $F_{1,21}=66.5$, $p<0.001$), which is consistent with data from other studies (Toro 2002, Maloy et al. 2003, Miranda 2004, Liu 2007). Egg diameter for 3 year-old *M. edulis* from Trinity was 54.5 ± 0.5 µm (mean ±SE) and 54.3 ± 0.6 µm from Long Harbour. Corresponding values for *M. trossulus* were 48.23 ± 1.4µm and 44.5 ± 0.5µm respectively. In 2004, egg diameter for 4 year old *M. edulis* from Long Harbour and Trinity was 62.5 ± 1.8µm and 54.6 ± 0.6µm respectively, and corresponding values for *M. trossulus* were 49.6 ± 0.8µm and 46.6 ± 0.8µm.

2.4. Discussion

At both sites, GSI values for both *M. edulis* and *M. trossulus* increased from May to July in 2003, peaking in August, and from May to June in 2004, declining after spawning to reach minimum values in November/ December in both years. This is broadly consistent with the spawning period identified by other studies on temperate *M.*

edulis, *M. trossulus* and *M. galloprovincialis* (Bayne and Newell 1983; Thompson 1984b; Rodhouse et al. 1985; Emmett et al. 1987; King et al. 1989; review by Seed and Suchanek 1992; Toro et al. 2002). During 2003 at the Trinity Bay site, a decline in GSI was not apparent in *M. edulis* or either hybrid group. Histological analyses confirmed that these genotypes had spawned, although a combination of spawning relatively fewer eggs or sperm coupled to the renewal of glycogen reserves in the mantle (Emmett et al. 1987) may have masked the effects of spawning on the GSI cycle. Additionally, in contrast to Toro et al. (2002) and in agreement with Maloy et al. (2003), there was little evidence of inter-specific differences in the spawning cycles that could act as a mechanism for maintaining reproductive isolation. Histological analyses and GSI data suggested that both F1 hybrid groups spawned a similar number of gametes over the same period as *M. edulis*, thereby increasing the likelihood of the production of viable hybrids (chapter 3). Furthermore, for logistical reasons, sampling intervals during spawning were not bi-weekly, and it is possible that there were spawning differences between species that could have been detected with greater sampling resolution.

Regardless of age, site, species or sex, at peak GSI mussels were not undifferentiated, spent, or in the early stages of development (King et al. 1989), and <10% of the mantle contained storage cells. Therefore, peak GSI or mantle (gonad) weight was a reliable indicator of reproductive output. This high degree of synchronization of gamete development may be attributable to the highly controlled conditions, since the mussels were of the same age, reared at each site in identical conditions and not prone to position effects (Okamura 1986; Seed and Suchanek 1992). The position of an individual mussel, e.g. within a matrix or clump, can strongly affect access to food or susceptibility to predation, thereby complicating any interpretation of data on allocation of resources to reproductive functions (Chapters 3, 4). Other studies have also reported a high degree of synchrony in the spawning of both males and females (Seed and Suchanek 1992; Toro et al. 2002; Lemaire et al. 2006), although spent mussels were often present in studies of sub tidal mussels (Rodhouse et al. 1985; Emmett et al.

1987; Seed and Suchanek 1992; Toro et al. 2002) and it is believed that males mature first (Kautsky 1982) and initiate spawning prior to females (Miranda 2004).

Peak GSI values were significantly lower in *M. edulis* than *M. trossulus*, although this did not reflect different stages of gamete development. For example, in agreement with Maloy et al. (2003), GVF values were not significantly different between species at peak GSI and visual assessment of development stages confirmed that gametes were well developed at peak GSI in both species. GSI differences between species were a direct result of increased allocation to the mantle for any given somatic tissue weight, and this was reflected in the greater gonad weight or potential reproductive output of *M. trossulus* standardized by body-size or soft tissue weight. The latter finding contrasts with Toro et al. (2002) at a productive sub-tidal environment (Thompson 1984a, b), where peak GSI between species was potentially similar in some contrasts, although sample sizes were small at each interval and age/ size or position effects (Bayne and Worrall 1980; Kautsky 1982; Bayne et al. 1983; Sprung 1983; Thompson 1984a; Rodhouse et al. 1985; Okamura 1986; Seed and Suchanek 1992) may have masked differences in mantle weight.

Diversion of resources to reproduction decreases the ability of bivalves to increase somatic production (Bayne and Worrall 1980; Kautsky 1982; Sprung 1983; Thompson 1984a; Rodhouse et al. 1985) or recover from spawning (Thompson 1984a), thereby influencing body condition (Toro et al. 2002; Lemaire et al. 2006). For example, in this study *M. trossulus* had a greater gonad weight than *M. edulis* at any given body-size. Consequently the peak GSI and subsequent decline in *M. trossulus* was greater than in *M. edulis*, albeit to a lesser extent in the second year of growth. This trend was reflected in the increased peak in, and slower recovery of, GSI and BCI. Furthermore, reproductive investment increases with age or size in bivalves (Bayne and Worrall 1980; Kautsky 1982; Thompson 1984b), and this strongly affected the amplitude of the decline and recovery of the GSI and BCI in all genotypes during the second year of growth.

Given the general life-history problem of how to allocate limited resources to reproduction, maintenance, growth and associated life-history traits (Stearns 1992), the observed differences in reproductive schedules between *M. edulis* and *M. trossulus* together with earlier maturation (Toro et al. 2002; Miranda 2004), may contribute to their co-existence. If so, the greater potential reproductive output of *M. trossulus* earlier in life likely enhances its colonization abilities or biotic potential, thereby offsetting the greater individual reproductive output and greater competitive ability associated with reproducing at a larger body size (Harger 1968, 1970; Seed and Brown 1978; Bayne and Newell 1983; Gardner and Skibinski 1990; Chesson 2000; chapters 3- 5).

Owing to potential cross-fertilization incompatibilities related to egg-size (Levitan 2005), selection may also favor divergence of egg size between species to minimize interbreeding. Whether or not the observed egg size differences have evolved to reduce hybridization thereby limiting interbreeding (Arnold 1997; Springer and Crespi 2007) is unclear. Certainly an argument exists that differences in egg size may limit hybridization in this system (Levitan 2005; David Innes, *pers. comm.* 2005). Despite the considerable differences in egg size between species observed in this and other studies (Toro et al. 2002; Maloy et al. 2003; Miranda 2004; Liu 2007), reproductively viable hybrids are produced in natural populations (Toro et al. 2004) and in laboratory crosses (Miranda 2004; Liu 2007) but occur at lower frequencies in natural populations than the parental species (Rawson 2003). Moreover, the production of many small eggs versus fewer large eggs could further enhance the biotic potential of *M. trossulus* and its subsequent ability to re-colonize disturbed environments, but it can do so only if the percentage of eggs developing into viable larvae is similar between species. To date studies on the relative viability of *M. trossulus* and *M. edulis* eggs are preliminary and require further investigation (Miranda 2004). Critically, egg size and body size differences may be common in hybridizing *Mytilus* species, with the larger species producing larger eggs (Secor and Hilbish 2001; Toro et al. 2002). Inter-specific differences in reproductive output were also more pronounced in the present study than in previous investigations

based on laboratory spawnings (Miranda 2004) or natural populations in Newfoundland (Toro et al. 2002), resulting in higher estimates of rates of population increase for *M. trossulus*. Mussels from Toro et al. (2002) were of unknown age, and sample sizes were small, both of which increase variation in fecundity estimates. Since mussels may spawn in holding tanks or during transit and may not release all their gametes in response to heat-shock or other stimuli, induction of spawning in the laboratory may also underestimate fecundity.

In conclusion, the observed differences between the reproductive schedules of *M. edulis* and *M. trossulus* help to explain the greater proportion of *M. trossulus* larvae settling at sites across Newfoundland (Comesaña et al. 1999; Miranda 2004; Toro et al. 2004; Stapleton 2007). These findings also lay the foundations for understanding the problem of species co-existence in this system. Trade-offs among growth, reproduction, and survival that give rise to life-history variation and consequently ecological differentiation offer an elegant and relatively unexplored solution to this problem in closely related, interbreeding species that compete for space and a shared resource (chapter 1.5-1.6). To this end, the study described in this chapter was complemented by three experiments to investigate allocation priorities in relation to resource availability, predator inducible defenses, and density dependent growth and survival in intra or inter-specific aggregations, the objective being to determine the potential for *M. edulis* and *M. trossulus* to co-exist while competing for space and other shared resources (see objectives 1.7, chapters 3-5).

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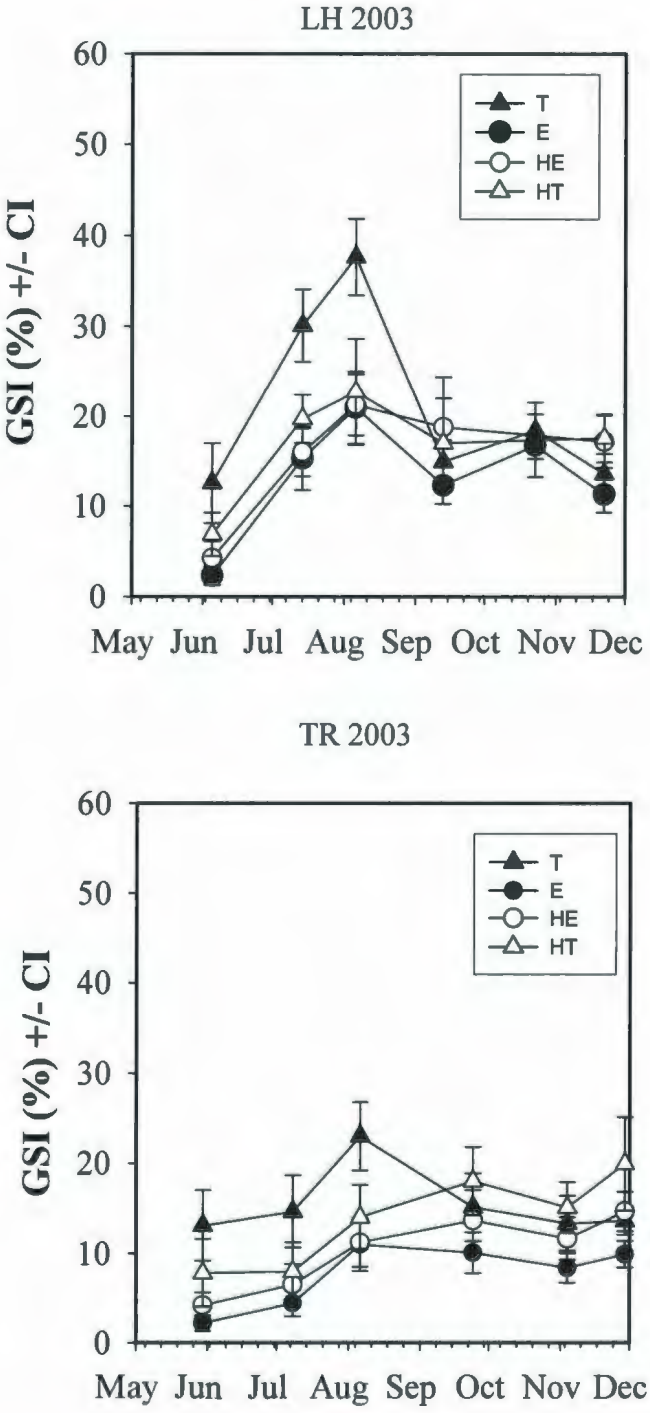

Figure 2.1. Seasonal variation in the gonado-somatic index (GSI) (mean % ± CI), expressed as the percentage of dried mantle: total dried soft tissues, during 2003 for *M. edulis* (E), *M. trossulus* (T), hybrid *edulis* (HE) and hybrid *trossulus* (HT) deployed at Long Harbour (LH) and Trinity (TR). Twenty to thirty mussels of each species were sampled monthly at each site over each 6 month sampling period.

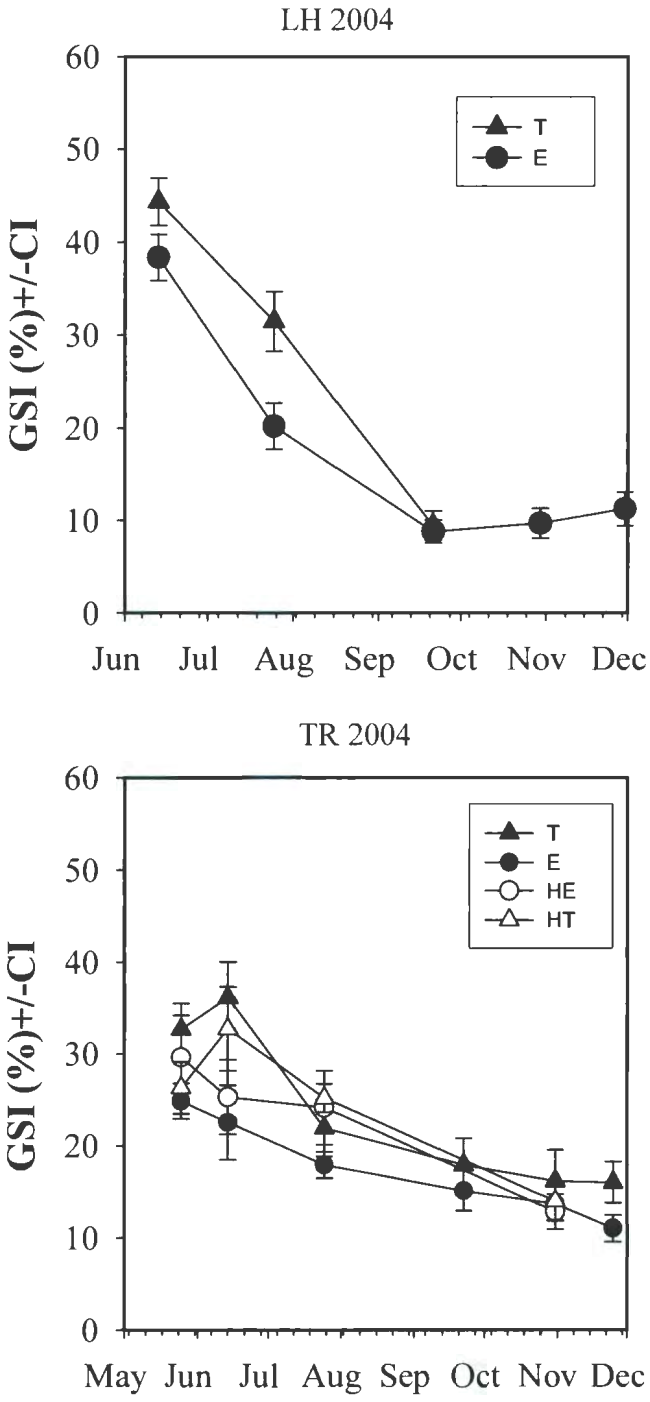

Figure 2.2. Seasonal variation in the gonado-somatic index (GSI) (mean % ± CI,), expressed as the percentage of dried mantle: total dried somatic soft tissues, during 2004 for *M. edulis* (E), *M. trossulus* (T), hybrid *edulis* (HE) and hybrid *trossulus* (HT) deployed at Long Harbour (LH) and Trinity (TR). Twenty to thirty mussels of each species were sampled monthly at each site over each 6 month sampling period.

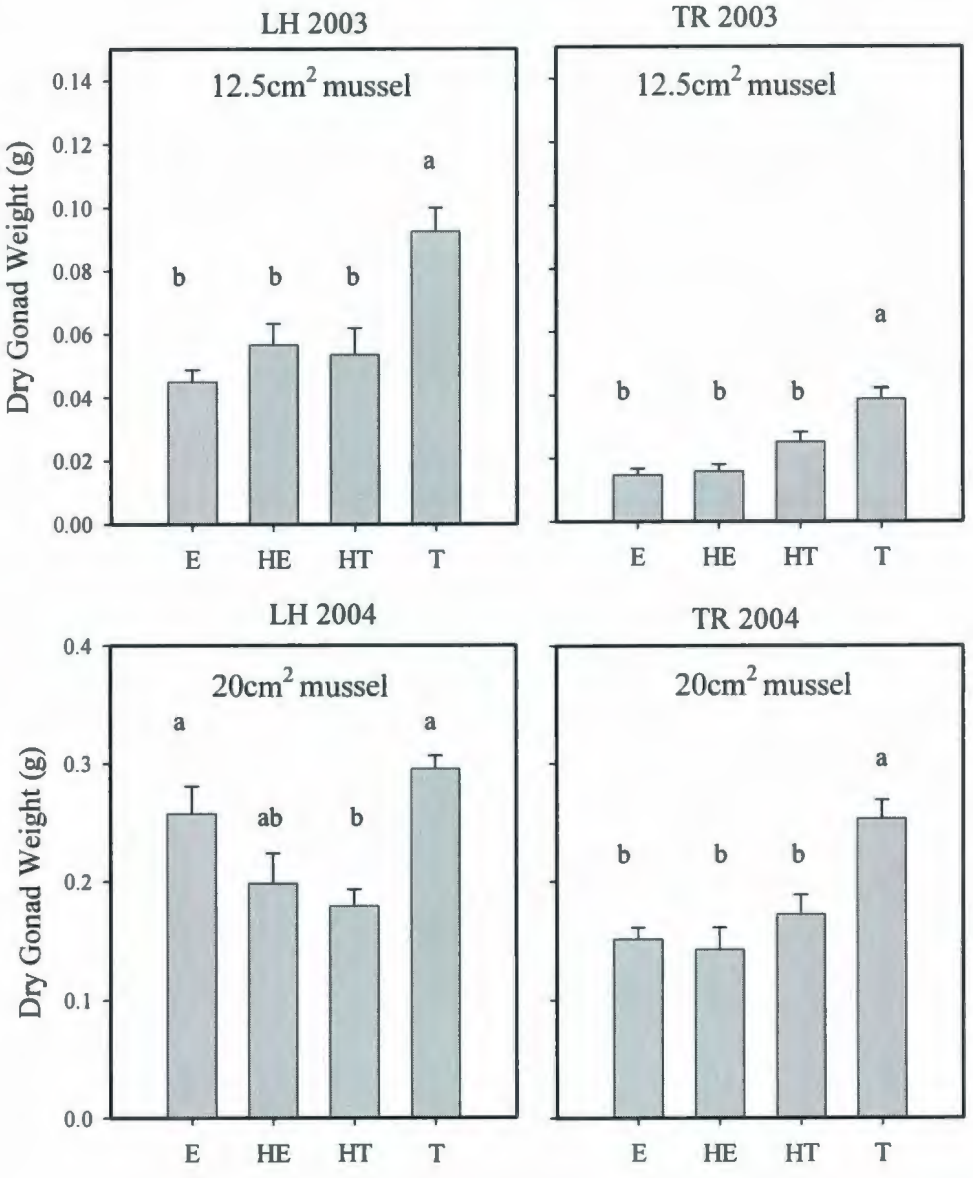

Figure 2.3. Dry gonad weight (g) (mean ± SE) standardized by shell surface area (12.5cm^2 in 2003 and 20cm^2 in 2004) of *M. edulis* (E), *M. trossulus* (T), hybrid *trossulus* (HT) and hybrid *edulis* (HE) from Long Harbour (LH) and Trinity (TR) at peak GSI in 2003 and 2004. Genotypes with the same letter were not significantly different from each other.

Table 2.1. One-Way ANCOVA (Unbalanced, Type III Sum Sq., General Linear Model) testing the effects of genotype (*M. edulis*, *M. trossulus*, hybrid *edulis*, hybrid *trossulus*) on gamete weight (expressed as ln x+1 dried gonad weight) adjusted by ln x+1 shell surface area. Mussels from Long Harbour and Trinity.

Gamete weight (dry gonad weight)				
Long Harbour 2003	Sum Sq	Df	F value	Pr(>F)
Genotype	2.0902	3	14.148	***
Surface area	1.2811	1	69.251	***
Residuals	2.7467	91		
Trinity 2003	Sum Sq	Df	F value	
Genotype	2.3396	3	11.248	***
Surface area	4.4392	1	64.030	***
Residuals	7.1410	103		
Long Harbour 2004	Sum Sq	Df	F value	
Genotype	0.5450	3	9.221	**
Surface area	4.6018	1	233.548	***
Residuals	1.8127	92		
Trinity 2004	Sum Sq	Df	F value	
Genotype	0.6407	3	7.350	**
Surface area	3.7068	1	127.573	***
Residuals	2.9056	100		

*** P<0.001, ** P<0.01

Chapter 3

To grow and survive or reproduce and die? Plasticity of somatic and reproductive traits and survival in hybridizing blue mussel species.

3.1. Introduction

Life-history theory tries to explain how natural selection acts on organisms to achieve reproductive success (Stearns 2000). In essence this is a problem arising from the allocation of limited resources, the solution to which may depend on the interplay of lineage specific (phylogenetic) constraints and "environmental variability" (Southwood 1977; Sibly and Calow 1986; Reznick et al. 1990; Stearns 1992, 2000; Charlesworth 1994; Roff 2001; Figure 3.1). Life-history trait trade-offs in turn arise from the proportion (prioritization) of limited resources allocated to maintenance, growth, defense and reproduction in fluctuating environments and the timing of these allocations (Fisher 1930; Williams 1966; Stearns 1992; Charlesworth 1994; Tilman 1994; Heino and Kaitala 1999; Roff 2002). Consequently, by preventing the evolution of Darwinian "demons" (individuals that develop and grow rapidly, reproduce continuously, and do not age), "life history trait trade-offs, through their effects on future reproductive success and competitiveness, are important determinants of species diversity and diversification" (Bonsall et al. 2004).

The absence of parental care or courtship in *Mytilus* species make them ideally suited for studying the relationship between allocation strategies, environmental variability (Thompson 1984a; MacDonald and Thompson 1985; Rodhouse et al. 1985; MacDonald and Bayne 1993; Jokela and Mutikainen 1995; Heino and Kaitala 1997; Bauer 1998; Stoeckmann and Garton 2001; Ernande et al. 2004) and the resulting life-history differentiation that allows co-existence between species (Figure 3.1). The blue mussels *Mytilus edulis* and *M. trossulus* are sibling bivalve species with divergent life-

histories (Toro 1999; Miranda 2004; chapters 2, 4-5) that co-exist and interbreed in the Northwest Atlantic hybrid zone (Comesaña et al. 1999; Miranda 2004; Toro et al. 2002, 2004). The species do not have different ingestion rates (Gardner and Thompson 2001; Miranda 2004) and growth, both in terms of shell and soft tissue, is similar up to maturity or spawning (chapter 2), implying similar net energy intake. Consequently, any observed allocation differences between the species may be attributable to the allocation of limited resources to a specific function.

The spatio-temporal abundance and potential for stable co-existence of *M. trossulus* and *M. edulis* in Newfoundland is not well understood (Toro et al. 2004). The distribution of *M. edulis*, *M. trossulus* and their hybrids across Newfoundland is patchy in space and time (Comesaña et al. 1999; Toro et al. 2004; Miranda 2004), with unexplained microscale intraspecific aggregations (Bates and Innes 1995; Miranda 2004) and no consistent niche partitioning along any obvious environmental gradient (Miranda 2004). Although a higher proportion of *M. trossulus* has been observed in the upper intertidal across California or at low salinity in both California (Braby and Somero 2006) and the Gaspé Bay (Qiu et al. 2002, but see Gardner and Thompson 2001; Moreau et al. 2005). Additionally, coexistence arising solely from "source" populations in Labrador dispersing to "sink" populations in Newfoundland (Miranda 2004) is also unlikely, due to the numbers of larvae and the distance that they would have to disperse to account for the observed distribution (Jerry Hilbish, *pers. comm.*). One striking feature of this hybrid zone is the decreased frequency of *M. trossulus* with increasing size class and a higher proportion of *M. trossulus* larvae than *M. edulis* in the water column (Comesaña et al. 1999; Miranda 2004; Toro et. al. 2004), potentially leading to increased reproductive investment and reduced survival (Toro et al. 2002) or growth (Miranda 2004) in *M. trossulus*. This implies that two or three-way allocation trade-offs may exist between reproduction and growth and/or survival and that the allocation choices differ among *M. edulis* and *M. trossulus*.

Allocation to traits associated with a large body-size earlier in life determines competitive ability (Harger 1968). Larger body size combined with thicker shells increases resistance to a predation from crabs or shell-boring predators (Seed and Brown 1978; Leonard et. al. 1999; Reimer and Harms-Ringdahl 2001), an important determinant of resistance (Southwood 1977; Petraitis 1995) to biotic disturbance in *Mytilus* species. Conversely, prioritizing reproduction early in life (Pianka and Parka 1975; Thompson 1984a) coupled with a small egg-size and high fecundity together constitutes a suite of colonization abilities (Kneitel and Chase 2004) that confer resilience (Southwood 1977) to biotic disturbance. Trade-offs among allocation to reproductive and somatic traits coupled to disturbance regimes (Shwilk and Ackerly 2005) can in turn establish successional dynamics that directly stabilize co-existence (Petraitis et al. 1989; Chesson 2000) or complement other stabilizing mechanisms (Yu and Wilson 2001; Levine and Rees 2002; Amarasekare 2003).

Because hybridization could result in the merging of the two species into one, displace one of the parental species, or create a third unique hybrid lineage (Arnold 1997), identifying barriers to hybridization is essential to understanding the future evolution of *M. edulis* or *M. trossulus*. Barriers to hybridization in this system (e.g. spawning asynchrony [Toro et al. 2002; chapter 2] or gamete incompatibility [Rawson 2003; Miranda 2004; Toro et al. 2004]) are generally incomplete. In combination, however, these barriers may explain the relatively lower frequency of hybrids, but there are caveats (see Toro et al. 2004). For example an improved understanding of life-history trait trade-offs in *M. edulis* and *M. trossulus* may elucidate the potential for spatial niche differentiation (Amarasekare 2003). Spatial niche differentiation in turn reduces hybridization, thereby determining the direction of selection for other barriers to hybridization. Furthermore, a potential post-zygotic barrier to hybridization, in terms of invasion success, is the relative viability and reproduction of F1 hybrids compared with the parental species. A study of resource allocation in *M. edulis* and *M. trossulus* and their hybrids would help to address post-zygotic barriers to hybridization.

An experiment to describe the plasticity of survival, somatic and reproductive traits in *M. edulis, M. trossulus* and their respective F1 hybrids is presented in this chapter. It specifically focuses on how the three traits respond to variability in site productivity within each genotype and the degree to which each trait differs among the genotypes. The resultant ecological differentiation and potential of the genotypes to co-exist are discussed. A companion experiment on the reproductive cycle was undertaken to determine peak gamete ripeness, fecundity and egg-size in the parental species. Two other companion experiments addressing the allocation patterns of *M. edulis* and *M. trossulus* in response to invertebrate predators and density dependent intra-specific or inter-specific interactions are referenced (chapters 3, 4).

3.2. Materials and Methods

3.2.1. Laboratory production of mussels, mussel sampling and dissection

Mussels (*M. edulis* and *M. trossulus*) were obtained from Bellevue (NL) and identified with two allozymes (MPI and EST-D), 4 nuclear DNA markers (ME, ITS, MAL and PLIIa) and one mitochondrial DNA marker (COIII) (Miranda 2004). Pure-species families (5 families per species) were then produced in the laboratory in July 2000. In addition, F1 hybrid families (5 families for each of the two reciprocal hybrid crosses) were produced from *M. edulis* females crossed with *M. trossulus* males (hybrid *edulis*) and *M. trossulus* females crossed with *M. edulis* males (hybrid *trossulus*). Pure species families and hybrid families were collectively referred to as "genotypes". Prior to deployment, all families within each genotype were pooled and reared in identical conditions for 3 years (Miranda 2004). Mussels were initially reared for 16 months in the laboratory, where they were fed a standard hatchery rearing diet consisting of mixtures of live algae and raw seawater in a partial recirculation system. They were transferred in November 2001 to a mussel farm in Notre Dame Bay for 12 months, after which they

were retrieved in November 2002, returned to the laboratory, and reared in the laboratory as described above. For the current experiment, two sites within Newfoundland were selected for mussel deployment in May 2003. Previous studies had demonstrated that Long Harbour (Placentia Bay) was an extremely productive site in terms of its capacity to support high growth rates (Miranda 2004), while Trinity (Trinity Bay) was deemed to be less productive after growth trials in 2002. As such, sites were treated as fixed effects rather than random effects (see analyses in sections 3.2.3- 3.2.5).

Mytilus species in temperate zones typically exhibit seasonal reproduction and growth fueled by the onset of the spring phytoplankton bloom (Thompson 1984b; Seed and Suchanek 1992). The 6-month sampling period coincided with the most productive growth phase (May- December) and spawning of gametes (chapter 2). To introduce a growth check before deployment, each individual mussel was marked 10mm from the shell margin with a Dremmel Tool™. During June 2003 ~200 individuals of each genotype were randomly assigned to 8 pearl nets (each genotype replicated) anchored off-bottom in strings of 4 arranged at random at each site. To prevent position effects, nets were rearranged at each sampling period. From June-December 2003 monthly samples were taken of 20-30 mussels per genotype per site. A strip of mantle tissue (~50mg wet weight) was excised from each mussel, fixed for 48 hours in Davidson's AFA solution and stored in 70% ethanol prior to histological analysis (chapter 2, section 2.2.3). Individuals were subsequently frozen at -20^{o} C to await removal of somatic and reproductive tissues.

3.2.2. Shell and soft tissue measurements

Figure 3.2 illustrates the gross anatomy of the two species. Shell length (anterior-posterior axis), height (dorso-ventral axis), width (lateral axis) and incremental growth (from the check mark) were measured for each mussel to the nearest 0.1mm with vernier calipers. The mantle, adductor, remaining soft tissue and shell were separated, dried to a

constant weight at 80° C, cooled in a desiccator and weighed to the nearest 0.0001g. Total dry weights of shell, somatic and somatic plus reproductive soft tissues, corrected for mantle tissue extracted, were calculated using weight loss in dried mantle tissue as an indicator of water content in the mantle pieces that were not dried (see the companion study on reproduction in chapter 2). The surface area of the shell was approximated using the formula:

$$A = l(h^2+w^2)^{0.5}0.5\pi \qquad \text{eqn 1}$$

Where A is surface area (cm^2), l is length (cm), w is width (cm) and h is height (cm) (Beadman et al. 2003). Because shell surface area is strongly correlated with shell volume, A is a reliable measure of body size (Freeman and Byers 2006).

3.2.3. Somatic allocation: Absolute growth and morphological traits

Quantification of somatic allocation encompassed growth of shell and somatic soft tissues or morphological traits (shell thickness and adductor mass corrected for body-size). Sexes were pooled because preliminary comparisons of shell and soft tissue parameters revealed no significant differences between sexes (data not shown). All analyses were conducted in "R" (R Project for Statistical Computing version 2.6.0) (Williams et al. 2000; Dalgaard 2002; Faraway 2002, 2004). A multivariate analysis was not adopted because it is not yet possible to combine binary (survival) and continuous data (somatic and reproductive traits) (Ernande et al. 2004; chapter 2; sections 3.2.3-3.25). Consequently, to reduce the probability of a Type 1 error when carrying out multiple comparisons from the same data-set, significance levels for all parameters used in the ANCOVAs were computed with the Bonferroni correction (Sokal and Rohlf 1995).

To compare somatic soft tissue production, final somatic soft tissue weight was regressed against initial somatic soft tissue weight (2003 data) and an ANCOVA carried out with genotype and site as fixed effects. The method used to calculate initial somatic

soft tissue weight is outlined in appendix 7.1. For tabular or graphical presentation, somatic soft tissue production was approximated as the final minus the initial value corrected for initial somatic soft tissue content. Soft tissue production (g) was converted to an energy equivalent (1g dry flesh weight = 21.8 kJ; Bayne et al. 1983). Further analyses of daily growth rates (shell length or somatic soft tissue weight) or growth of somatic soft tissues corrected for initial shell length demonstrated that these tests were reliable (data not shown). For shell area growth, it was not possible to estimate initial shell surface area for each individual in the final sample during 2003 so growth rates were estimated after regression of shell surface area against time (days). Sampling intervals were treated as a continuous explanatory variable (days) in a regression based analyses rather than a fixed factor in an ANOVA (Sokal and Rohlf 1995; Dalgaard 2002). The transition from an ANOVA to ANCOVA in this instance is a form of model simplification that accommodates multiple measurements of y (the response variable) for each value of x (the independent variable). Accordingly, the error associated with the "deviance" from regression was computed before the minimally adequate statistical contrasts were made. In all instances the variation attributable to the "deviation" from the regression term was not significant and was removed from the model using the step function in R. Shell area growth rate coefficients, i.e. the slopes of the regression of body-size with time for each genotype at each site, were compared by ANCOVA (homogeneity of slopes model) with genotype and site as fixed effects. For graphical presentation the regression coefficients i.e. rates of change in shell area with time (shell area growth rate) were plotted for each genotype within each site.

Net effects were non-significant in all ANCOVA's and were therefore dropped from the analysis. All tests conformed with the assumptions of normality and homogenous distribution of residuals required for the General Linear Model. For accurate interpretation of the results (Day and Quinn 1989), Bonferroni adjustments were applied to "a priori" contrasts in absolute growth among genotypes at each site and within genotypes between sites (Table 3.1). For the latter, if absolute growth was significantly

different between sites for any given genotype, flexibility of allocation to absolute growth was assumed.

A heavier shell or adductor for a given surface area effectively represents a "thicker shell or stronger adductor" (Beadman et al. 2003, Freeman and Byers 2006), while standardizing muscle components by surface area rather than shell length eliminates bias arising from species specific differences in shell width or height. Genotypic differences in these morphological traits were compared by ANCOVA with genotype, site and final sample as fixed effects and shell surface area as a covariate. Bonferonni adjusted "a priori" pair-wise contrasts were undertaken as follows: i) Among genotype comparisons within each site separately; ii) Between site comparisons within genotypes.

3.2.4. Survival

All mussels were held in nets off-bottom away from predators, and nets were cleaned monthly to remove fouling organisms and maintain optimal conditions for growth and reproduction. The observed mortality is therefore directly attributable to physiological stress only. Survival of each genotype at each site during 2003 (computed by the Kaplan-Meier estimator) was compared using the log-rank test (Dalgaard 2002). Bonferroni corrected pair-wise contrasts were as follows: i) Among genotype comparisons within each site separately for each sample; ii) Between site comparisons within genotypes (Table 3.2).

3.2.5. Allocation to reproduction

A detailed analysis of gamete output and the spawning cycle is presented in chapter 2. These results were integrated to enable calculation of a suitable index of reproductive effort for each genotype. The gonado-somatic index (GSI) cycle indicated

that mussels had attained peak ripeness at both sites in August 2003, and this was confirmed by subsequent histological analysis (chapter 2). Because mantle weight is a reliable indicator of reproductive output in ripe mussels from Bellevue, NL (Toro et al. 2002; chapter 2), a simple dry weight reproductive effort index was estimated as follows:

$$RE = \frac{P_r}{P_g + P_r} \qquad \text{eqn 2}$$

Where RE = reproductive effort, P_r = dry mantle weight and P_g = dry weight of somatic soft tissue (Thompson 1984a). This index did not vary strongly with size within species (initial shell length or body weight) or among sexes. Furthermore, when dry gamete weight was substituted for dry mantle weight and the data analyzed for each sex separately, the results were consistent with the above index (data not shown). Reproductive effort data for each year class were analysed by ANOVA after arcsine-square root transformation, with genotype and site as fixed effects (Type III SS). Bonferroni corrected tests (Type III SS) were used for "a priori" pair-wise contrasts where significant genotype effects were obtained.

3.3. Results

3.3.1. Somatic allocation: Absolute growth and morphological traits

M. edulis allocated significantly more resources to shell or somatic soft tissue growth at both sites than did *M. trossulus* (Figure 3.3, Table 3.1). Unlike *M. trossulus*, *M. edulis* also grew significantly faster at Long Harbour than at Trinity i.e. flexible allocation to shell and somatic soft tissues was evident. Hybrid *edulis* shell surface area growth was also significantly greater at Long Harbour than Trinity, although growth of somatic soft tissues was not. At both sites, there was no difference in shell or soft tissue growth among *M. edulis* and hybrid *edulis*. Somatic growth was also significantly lower in hybrid *trossulus* than in *M. edulis* at Long Harbour, but not significantly different at Trinity. Hybrid *trossulus* was also similar to *M. trossulus* in that growth did not increase

at Long Harbour compared with Trinity, i.e. no "flexible" allocation to somatic growth (shell surface area and soft tissues) was evident. Shell growth, however, was significantly greater in hybrid *trossulus* than in *M. trossulus* at both sites. Furthermore, soft tissue growth in hybrid *trossulus* was significantly greater than in *M. trossulus* at Trinity but not significantly different from *M. trossulus* at Long Harbour. On the other hand, somatic growth in hybrid *edulis* was significantly greater than in *M. trossulus* at both sites. Allocation to somatic growth, both shell surface area and soft tissues, among hybrid groups were not significantly different at either site. Furthermore, variability in each somatic trait was much greater in the hybrid groups than in *M. trossulus* or *M. edulis*.

By the end of the 6 month deployment period the shell and adductor (data corrected for shell surface area) were approximately 20 or 50 % heavier in *M. edulis* than in *M. trossulus* at Long Harbour or Trinity (Long Harbour; shell $F_{1,55}$=40.9, $p<0.001$; adductor $F_{1,55}$=40.9, $p<0.001$: Trinity; shell $F_{1,55}$=16.9, $p<0.01$; adductor $F_{1,55}$=40.9, $p<0.001$). The shell and adductor muscle weights for the F1 hybrid genotypes in the final sample from Long Harbour or Trinity Bay were also not significantly different from those in *M. edulis* but were significantly greater than in *M. trossulus* (data corrected for shell surface area).

3.3.2. Survival

The survival of *M. trossulus* and hybrid *edulis* was significantly lower at Long Harbour than Trinity, while the survival of *M. edulis* and hybrid *trossulus* remained unchanged among the sites (Figure 3.4, Table 3.2). Within each site, survival was not significantly different between *M. edulis* and *M. trossulus*. At both sites, hybrid *edulis* had significantly lower survival than both *M. edulis* and *M. trossulus*. Furthermore, survival in hybrid *trossulus* was not significantly different than in any of the other genotypes, although variation was much greater in hybrid *trossulus*.

3.3.3. Allocation to reproduction

For all genotypes, reproductive effort was significantly greater at Long Harbour than Trinity i.e. flexible allocation to reproductive effort was evident (Figure 3.4, Table 3.2). Moreover, in contrast to somatic traits and survival, reproductive effort was significantly greater in *M. trossulus* than *M. edulis* at both sites. The difference between the species was greater at Long Harbour than at Trinity, as it was for somatic investment. At both sites reproductive effort was not significantly different between *M. edulis* and hybrid *edulis,* but was significantly lower than in *M. trossulus,* while the reproductive effort of hybrid *trossulus* was significantly lower than that of *M. trossulus* and not significantly different from *M. edulis*. Reproductive effort did not differ significantly among F1 hybrid groups.

3.4. Discussion

Flexible allocation to both growth and reproductive effort, which were both greater at Long Harbour than Trinity, was apparent in *M. edulis*. At the same time, *M. edulis* survival was not influenced by variation in site productivity. In contrast, *M. trossulus*, and to a much lesser degree hybrid *edulis*, responded to the conditions in Long Harbour by increasing reproductive effort and maintaining growth constant, at the expense of survival, whereas hybrid *trossulus* also increased reproductive effort and maintained growth at Long Harbour relative to Trinity, but with no change in survival. Variation in survival and growth, though, was much greater in F1 hybrids than in either *M. edulis* or *M. trossulus*, while hybrid *edulis* survival was lower than both *M. edulis* and *M. trossulus.* If the proportion of resources allocated to the different traits were to remain unchanged, dispersal to a more productive environment would result in an increase in every trait. Evidently the pattern of flexibility is constrained by physiological trade-offs (see Ernande et al. 2004). Clearly the "form" of these trade-offs differs between *M. edulis* and *M. trossulus, M. edulis* prioritizing allocation to somatic traits to a greater extent than *M. trossulus,* while *M. trossulus* prioritizes reproductive traits to a greater extent than *M.*

edulis. In *M. trossulus* the active shift in resource allocation likely enhances present rather than future reproduction (Pianka and Parka 1975 Stearns 1992, 2000), whereas in *M. edulis* the opposite holds (Pianka and Parka 1975). Since reproduction can be traded-off with survival (linked to maintenance) and/or growth (Stearns 1992), information on reproductive trade-offs can also be gleaned from between-genotype comparisons within each site (Jokela and Mutikainen 1995; Stoeckmann and Garton 2001; Ernande et al. 2004). Relative to *M. edulis*, *M. trossulus* increased reproductive effort and reduced somatic soft tissue production at each site. Increasing the former leads to a decrease in the latter. Since there was no reason to believe that ingestion rates and therefore energy intake were different among species (Gardner and Thompson 2001; Miranda 2004; chapter 2), this finding provides further evidence of a species-specific trade-off between reproduction and growth.

Overall, *M. trossulus* and *M. edulis* exhibit characteristics of relatively shorter and long-lived species respectively. Life-history theory predicts that species that are short-lived can improve fitness through greater reproductive investment earlier in life (eventually at the expense of reproductive lifespan), whereas potentially long-lived species may gain fitness through an extended reproductive life-span (Kirkwood and Holliday 1979; Stearns 1992, 2000; Roff 2001) and/ or by increasing allocation to growth and storage (Kozlowski 1991). Compared with *M. trossulus, M. edulis* prioritizes somatic growth in natural populations on the seabed (Toro 2002; chapter 5) or in suspended culture (Mallet and Carver 1995; Miranda 2004; Penney et al. 2002, 2006, 2007), at variable densities in both intra/ interspecific clumps, and when threatened by invertebrate predators (chapter 4). Conversely *M. trossulus* prioritizes reproduction in natural sub-tidal populations and in suspended culture (personal obs.), as well as at low food levels in the laboratory (Miranda 2004). It spawns smaller eggs (Toro 2002; Miranda 2004; chapter 2) and matures earlier (Toro 2002) than *M. edulis*. Because care was taken to account for variability arising from position effects, age, density, and exposure in this study and predator or density dependent intra/ interspecific interactions in companion

studies (chapters 4- 5); a distinct set of allocation strategies was evident. Interspecific differences in somatic and reproductive traits are partly attributable to differences in responses to changes in resource availability and/ or density (chapter 5). This likely reflects a differential response to "resource pulses" (e.g. increased food availability or release from competition after gap formation following disturbance in mussel beds; Seed and Suchanek 1992; Petraitis 1995; table 3.3), whereby *M. edulis* exploits these "resource pulses" by growing to a larger size while *M. trossulus* increases its colonization abilities by prioritizing reproductive traits. Residual intra-specific variability may be attributable to limited introgression in combination with unexplained measurement error and genetic variation, while the high variability of F1 hybrid traits perhaps reflects the conflicting allocation "strategies" of the parent genotypes.

The different allocation strategies of *M. edulis* and *M. trossulus* pave the way for an original explanation of the maintenance of species diversity within this hybrid zone. Traditional explanations of the assembly, dynamics and structure of ecological communities include: i) intricate trade-off based theories of stabilizing niche differentiation, arising from individual-level constraints within an environmental context, that, although well supported (Amaraskaree 2003; Kneitel and Chase 2004; Shea et al. 2004; Schwilk and Ackerly 2005), may not predict limits to species diversity (Kinzig et al. 1999; Hubbell 2001; Tilman 2004; Adler et al. 2007; Table 3.4) or adequately accommodate intraspecific variability (Chave 2004); ii) neutral theories (Hubbell 2001; Table 3.4) centered on stochastic drift and equalizing mechanisms (Hubbell 2001, Condit et al. 2006), that, although influential in developing quantitative models in molecular evolution (Chave 2004), are "currently blind to the numerous biological stabilizing mechanisms that may contribute to niche differentiation in real communities" (Brown et al 2001; see also Chesson 2000; Chave 2004; Tilman 2004). To address these shortcomings, integrating aspects of both approaches has recently been favored (Tilman 2004; Kneital and Chase 2004; Bonsall and Mangel 2004; Bonsall et al. 2004; Gravel et al. 2006; Chesson 2000, 2007; Nekola and Brown 2007; Clark et al 2007; Adler et al.

2007) e.g. by combining stochastic demographic extinction with equalizing and stabilizing mechanisms. Moreover, because model communities are more diverse when they exhibit life-history trade-offs, and aspects of community dynamics can be traced to certain trade-offs (Pacala et al. 1996; Chave 1999; Chave et al. 2002; Harpole and Tilman 2007), understanding the life history "strategies" of interacting species may help to unlock the appropriate approach. For example, in *Mytilus* species a large body size increases overall fecundity (Bayne and Newell 1983; Gardner and Skibinski 1990) and resistance to predation (Seed and Brown 1978), which may confer a competitive advantage (Harger 1968). By virtue of an allocation trade-off prioritizing somatic production early in life, *M. edulis* rapidly attains a larger body size, potentially suppressing smaller *M. trossulus* at high densities (chapter 5). The production of thicker shells, supported by stronger adductor muscles, further increases resistance to predators (Leonard et al. 1999; Figure 3.2). In *M. trossulus* a trade-off prioritizing reproduction, coupled with earlier maturation (Toro et al. 2002; Miranda 2004) and larger numbers of smaller eggs (Toro et al. 2002; chapter 2), enhances its colonization abilities and resilience to disturbance. Furthermore, *M. trossulus* may buffer negative inter-specific interactions by reducing average terminal body size and life-span (chapter 5), can colonize any recently disturbed patch regardless of *M. edulis* presence (Miranda 2004), and displacement of *M. trossulus* from mixed species clumps is not instantaneous (chapter 5). Consequently, despite the apparent competitive asymmetry (chapter 5), a strict competition colonization trade-off (Tilman 1994) may not occur (Table 3.4). Rather *M. edulis,* because of its resistance to predators, may ultimately succeed *M. trossulus* by interference and/or replacement competition (Calcagno et al. 2006), thereby explaining the decrease in the frequency of *M. trossulus* with increasing size (Miranda 2004; Toro et al. 2004).

These species-specific two or three-way trade-offs between displacement and/or replacement competition and colonization abilities not only equalize fitness differences between species (Chesson 2000) but may also drive a successional process in each

locality (Chesson 2000; Table 3.4), a mechanism commonly invoked to explain the stabilization of the co-existence between sessile species (Tilman 1994; Holmes and Wilson 1998; Pacala and Rees 1998; Yu and Wilson 2001; Levine and Rees 2002; Calcagno et al 2006). Moreover, the prevailing disturbance regime (Table 3.3) likely leaves localities vacant randomly in space and time, ensuring a mosaic of successional states within a spatial-temporal "landscape" (Chesson 2000) in which diversity is maximized at intermediate values of disturbance frequency (Petraitis et al. 1989; Chesson 2000, Shea et al. 2004). If this "landscape" is punctuated with sustained departures from an intermediate disturbance regime such that both species are favored in different parts of the landscape, stabilizing spatial storage effects could also emerge (Chesson 2000; Amarasekare 2003; Snyder and Chesson et al. 2004; Table 3.4). For example, relative to *M. edulis*, *M. trossulus* could proliferate in upper intertidal or low salinity environments where invertebrate predation is less frequent (Seed and Suchanek 1992; Reimer and Harms-Ringdahl 2001). On the other hand, *M. edulis* would dominate in areas of sustained selective invertebrate predation (Miranda 2004; chapter 5) and out-compete *M. trossulus.* Settlement from areas that favor one or the other species may in turn reinforce populations where the two species are co-existing through successional dynamics.

Although there are multiple allocation trade-offs (Figure 3.1) that may explain the co-existence of other groups of interacting *Mytilus* species (see for example Erlandsson et al. 2006), they essentially confer resilience (ability to recolonize) or resistance to biotic or physical disturbance. In the Atlantic *Mytilus edulis- trossulus* hybrid zone successional dynamics coupled to spatial storage effects (Chesson 2000; Amarasekare 2003; 2004) could help to explain the patchy mosaic distribution, intra or interspecific clumping patterns, or differentiation in intertidal or low salinity environments (Bates and Innes 1995; Qiu et al. 2002). By preferring *M. edulis*, which is more resistant to grading, socking, and low density invertebrate predation, the well established and intensive aquaculture industry in Prince Edward Island and parts of eastern Newfoundland, may be disrupting the equilibrium of forces maintaining the hybrid zone.

These co-existence mechanisms may also in turn facilitate hybridization where clumps of mussels in intermediate successional states contain mixtures of the two species, or where source-sink dynamics are established by spatial storage effects. Unlike situations in which the species intermix without any niche differentiation, however, niche-based stabilizing mechanisms that concentrate intra- relative to inter-specific interactions do limit the overall potential for hybridization (Bierne et al. 2002, 2003, 2006). By enhancing traits associated with either resilience or resistance to disturbance an organism must express key life-history traits (e.g. egg-size and number) in such a way that hybridization is likely to be reduced (Innes, D. J., *pers. comm.*). When hybridization does occur, hybrid *edulis* is ecologically similar to *M. edulis* in that it prioritizes allocation to increasing its body size or strengthening its defensive traits. Hybrid *trossulus*, like *M. trossulus*, did not prioritize allocation to absolute growth, although it generally maintained a higher somatic investment (at the expense of reproduction) than *M. trossulus*, and most defensive traits adjusted for body size were more similar to those of *M. edulis*. Despite these similarities the costs in terms of flexible allocation to reproductive investment or survival were greater in both hybrid groups, while the greater absolute growth with increased resource availability compared with hybrid *trossulus* would leave *M. edulis* less vulnerable to predation. Furthermore, because established species or individuals should more strongly inhibit invaders more similar to themselves (Dieckmann and Doebeli 1999, 2000; Tilman 2004), and because numbers of propagules released and establishment success are well correlated (see Tilman 2004 and citations therein), the generally reduced viability of the F1 hybrids in terms of flexible allocation to growth and reproduction could then decrease their establishment success.

In conclusion, genotype specific allocation trade-offs coupled to flexible allocation that during "resource pulses" enhance the colonization abilities of *M. trossulus* or the competitive and replacement abilities of *M. edulis* may simultaneously equalize fitness between species and strengthen stabilizing co-existence mechanisms. Together

with the apparently decreased fitness of F1 hybrids, these stabilizing mechanisms may reduce the rate of hybrid production by increasing intra-specific relative to inter-specific interactions, while the divergent allocation strategies may drive the expression of key life-history traits, resulting in phenotypic barriers to hybridization between species.

3.5. References

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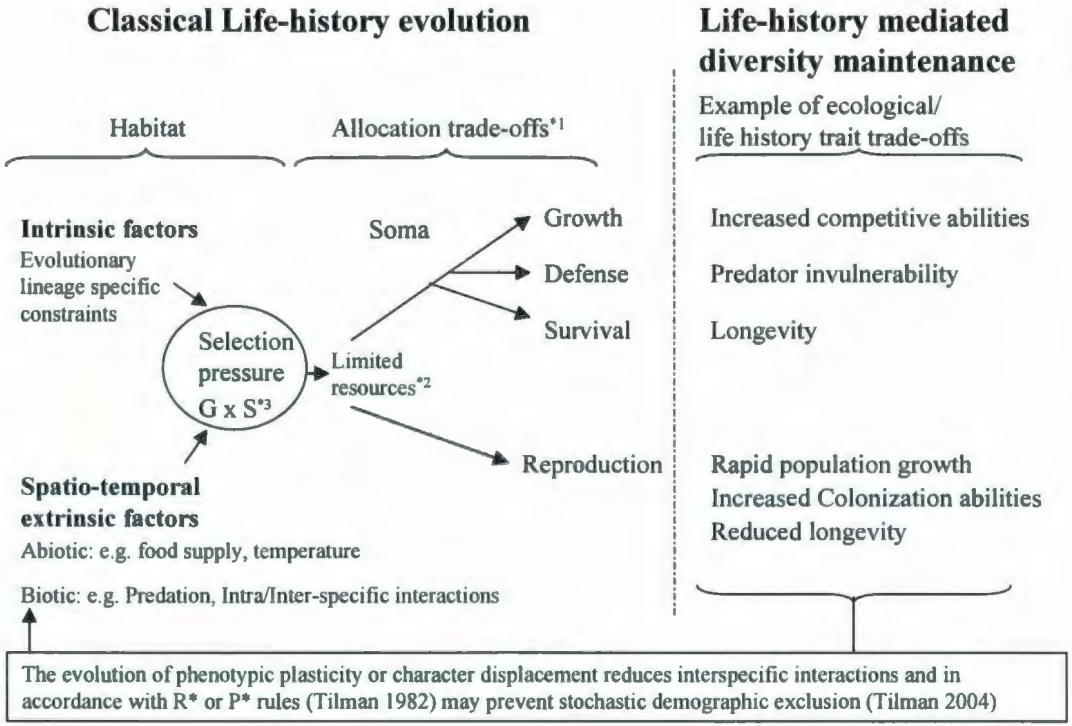

*1. Allocation trade-offs enhance diversity alongside other stabilizing niche-based mechanisms and maybe directly attributable to diversity, depending on the degree of asymmetry between the life-history traits of interacting species (e.g. Calcagno et al. 2006).
*2. The timing of and form of the allocation trade-off give rise to key life-history traits: e.g. life span, age and size at maturity egg size and number.
*3. Selection pressure in habitat e.g. G x S where G= growth promoting (high / low) and S= ratio of juvenile: adult survival (high/low) [Sibly and Calow 1986].

Figure 3.1. Framework for understanding the evolution of life-history mediated diversity maintenance. Intrinsic and extrinsic environmental factors drive the evolution of optimal or evolutionary stable resource allocation strategies, resulting in local maxima for populations in variable environments. These resource allocation strategies are manifest as ecological or demographic trade-offs that may evolve to reduce the risk of intra or inter-specific interactions resulting in stochastic demographic exclusion of distinct phenotypes or species. The bio-diversity considered here is more applicable to guilds of closely allied individuals sharing similar resources. However many such guilds could co-exist in a complete community, one for every resource for instance (*sensu* Calcagno et al. 2006).

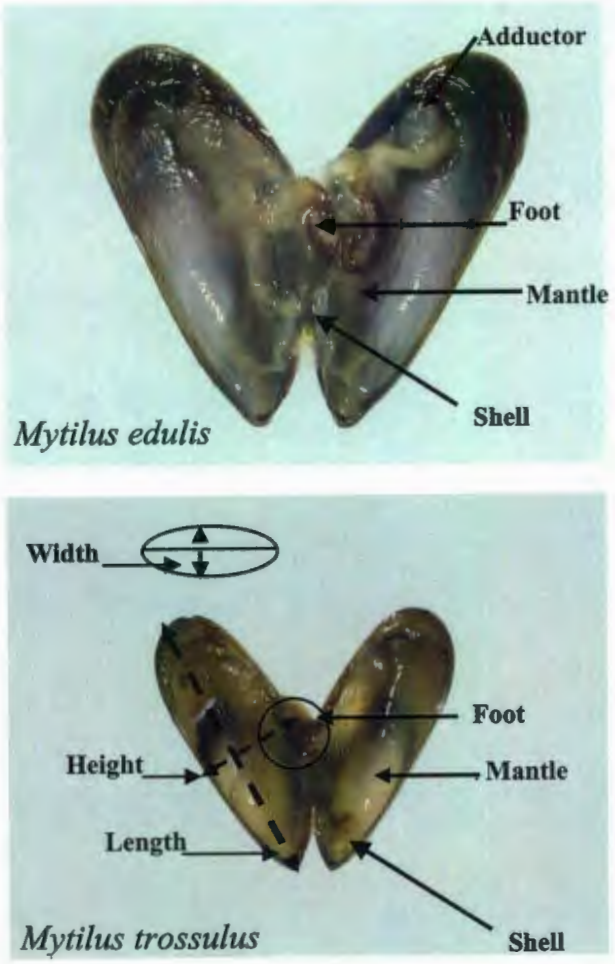

Figure 3.2. Morphological features of 3 year old *M. edulis* and *M. trossulus* from Long Harbour. During the reproductive period the mantle was composed mostly of gametes (chapter 2, section 3.3.3). The more developed mantle of *M. trossulus* relative to *M. edulis* partially obscured the smaller adductor and smaller more translucent foot. *M. edulis* attained a much larger body size, invested more energy in somatic soft tissues and developed a thicker shell than *M. trossulus* (section 3.3.1).

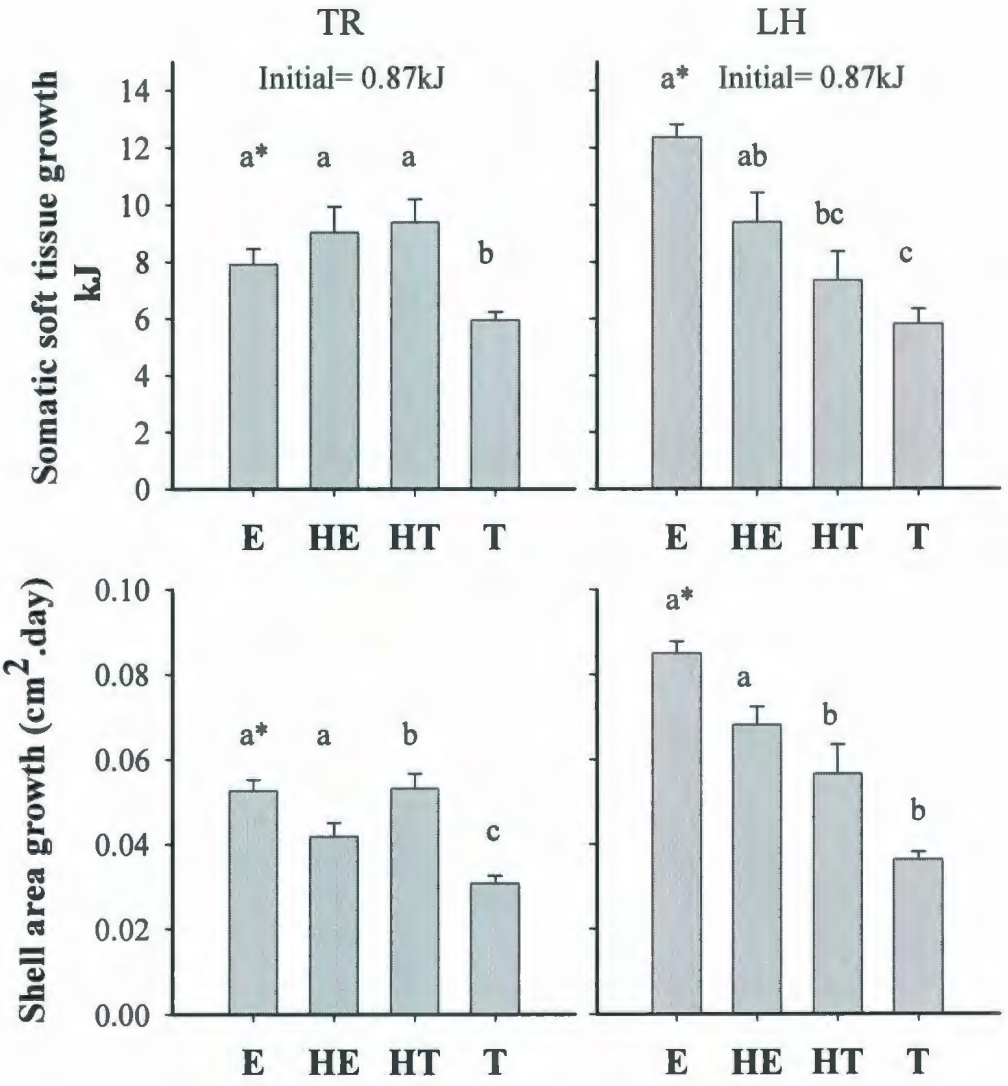

Figure 3.3. Growth of somatic soft tissues (kJ) (corrected for initial soft tissue content (kJ)) and daily shell area growth (cm^2) during May-December 2003 (170 days) for *M. edulis* (E), *M. trossulus* (T), hybrid *trossulus* (HT), and hybrid *edulis* (HE) deployed in Trinity (TR) and Long Harbour (LH). Error bars represent ± 1 standard error. Genotypes with the same letter were not significantly different. "*" denotes a significant between site contrast for each genotype separately (Table 3.1).

Table 3.1. Pair-wise contrasts of soft tissue growth (Final soft tissues (kJ) regressed against initial soft tissues (kJ)) and shell surface area growth co-efficients (slopes of shell surface area regressed against time days) for *M. edulis* (E), *M. trossulus* (T), hybrid *trossulus* (HT), and hybrid *edulis* (HE) deployed in Trinity (TR) and Long Harbour (LH).

		Soft tissue growth			Shell area growth		
		df	F	P	df	F	P
LH	E:T	1,44	82.5	***	1,299	457.7	***
	E:HT	1,31	17.5	**	1,247	61.9	***
	E:HE	1,41	2.5		1,287	1.1	
	T:HT	1,29	3.8		1,222	13.2	
	T:HE	1,39	14.4	**	1,262	228.9	***
	HE:HT	1,26	2.2		1,210	38.4	***
TR	E:T	1,45	36.3	***	1,288	284.1	***
	E:HT	1,36	15.0	**	1,276	74.6	***
	E:HE	1,39	0.5		1,301	0.0	
	T:HT	1,37	47.3	***	1,280	17.0	***
	T:HE	1,40	28.4	***	1,305	205.0	***
	HE:HT	1,31	3.8		1,293	64.1	***
Between	E	1,45	41.9	***	1,304	26.3	***
LH-TR	T	1,44	0.4		1,283	3.4	
	HE	1,35	3.3		1,284	3.2	
	HT	1,22	2.8		1,219	2.2	

** P<0.01, *** P<0.001

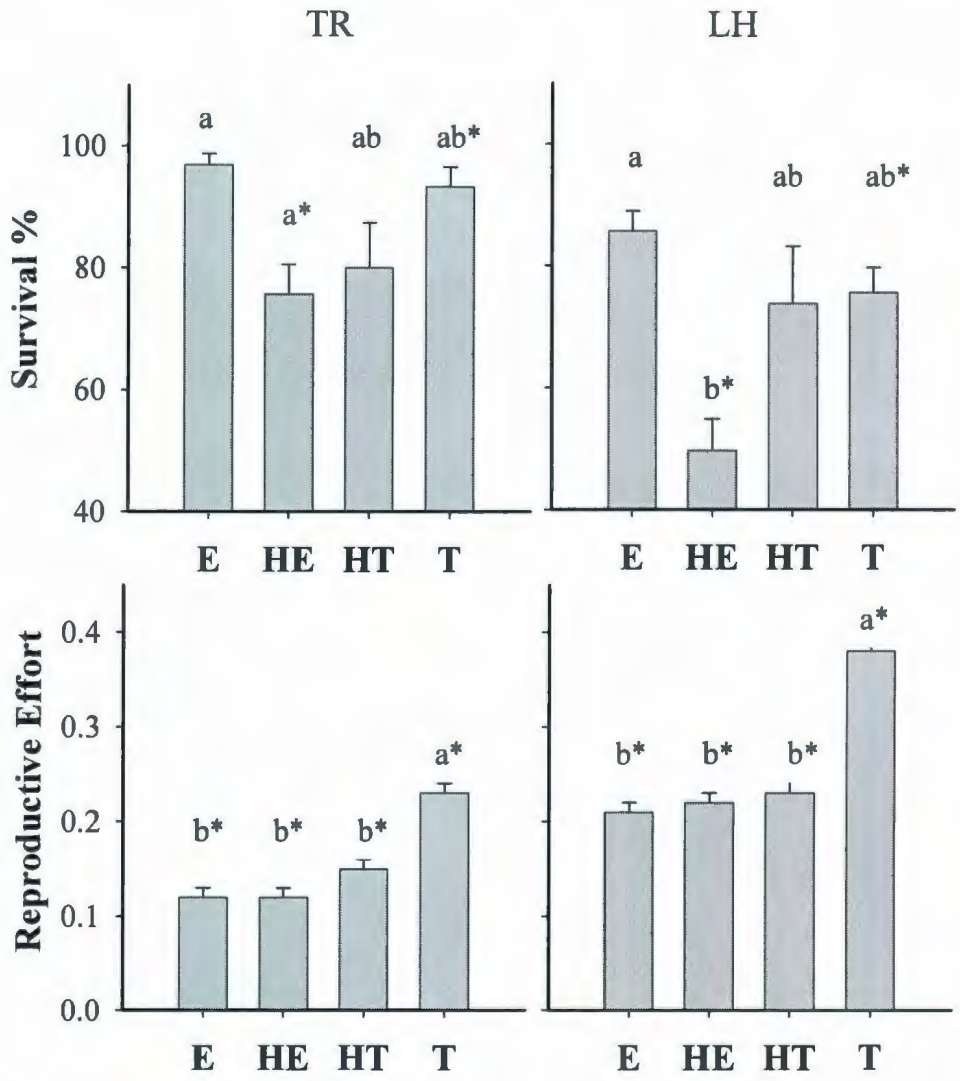

Figure 3.4. Survival and reproductive effort (RE) during May- December 2003 for *M. edulis* (E), *M. trossulus* (T), hybrid *trossulus* (HT), and hybrid *edulis* (HE) deployed in Trinity (TR) and Long Harbour (LH). Error bars represent ± 1 standard error. Genotypes with the same letter were not significantly different. "*" denotes a significant between site contrast for each genotype separately (Table 3.2).

Table 3.2. Pair-wise contrasts following logistic regression (survival) or ANOVA (reproductive effort) in *M. edulis* (E), *M. trossulus* (T), hybrid *trossulus* (HT), and hybrid *edulis* (HE) deployed in Trinity (TR) and Long Harbour (LH).

		Survival		RE		
		Chisq	P	df	F	P
LH	E:T	2.3		1,52	78.1	***
	E:HT	1.0		1,39	0.3	
	E:HE	35.0	***	1,53	0.1	
	T:HT	0.1		1,39	38.2	***
	T:HE	26.4	***	1,53	86.4	***
	HE:HT	8.5	_	1,40	0.6	
TR	E:T	0.3		1,56	53.8	***
	E:HT	5.8		1,48	3.4	
	E:HE	14.7	**	1,56	0.0	
	T:HT	4.1		1,48	23.6	***
	T:HE	12.7	*	1,56	51.2	***
	HE:HT	2.2		1,48	3.0	
Between	E	8.0	_	1,54	46.4	***
LH-TR	T	16.0	***	1,54	62.3	***
	HE	34.6	***	1,55	41.9	***
	HT	5.9		1,33	14.9	**

* P<0.05, ** P<0.01, *** P<0.001

Table 3.3. Sources of abiotic and biotic disturbance in Newfoundland.

Biotic/Abiotic	Disturbance classification	Indiscriminate?	Frequency	Assumptions
Abiotic	Storms	Scale dependent	~5 per year	Minor: Generally *Mytilus edulis* > *M. trossulus* [1]
	Ice bergs/ ice pack	Yes	Annual	Multiple pieces of pack ice scours large areas
	Freshwater run-off or ice melt, heavy rainfall	No	~5 per year	Provides temporal refugia from invertebrate predators concentrating *Mytilus trossulus* relative to *M. edulis* [2]
Biotic	Settlement (selective)	Scale dependent	Annual	Topside settlement of *Mytilus trossulus* [3]
	Predation	Scale dependent	Seasonal	Generally *Mytilus trossulus* >*M. edulis* [4]
	Post spawning mortality	No	Annual	*Mytilus trossulus* > *M. edulis*
	Interspecific interactions	No	Continuous	*Mytilus trossulus* life-history mediates negative interactions leading to gradual dominance of *M. edulis* (chapter 5)
	Intraspecific interactions	No	Continuous	*Mytilus edulis* > *M. trossulus* (chapter 5)
	Anthropogenic (culture)	No	Continuous	Concentrates *Mytilus edulis* relative to *M. trossulus* [5]

1. The shells of *M. trossulus* are narrower and more elongated potentially decreasing susceptibility to wave action.
2. Low salinity may i) Decrease invertebrate predation leading to greater survival of *M. trossulus* ii) Confer a physiological advantage leading to increased larval development.
3. Settlement at different depths is sometimes evident (Kenchington et al. 2002; Freeman and Macquarrie 2002; Miranda 2004). It is hypothesized that clumping with conspecifics is advantageous *to M. trossulus* in that it could avoid smothering, in a manner akin to *M. edulis* clumping with *M. californianus* (Harger 1968), and as observed in *M. edulis*/ *M. californianus* may destabilize clumps during storms if *M. trossulus* aggregates on top of *M. edulis* (Seed and Suchanek 1992). Alternatively topside settlement on collector ropes could be attributed to freshwater runoff (see 2).
4. Perhaps so intense that it resets the successional process as all individuals below a size refuge are culled (Leonard et al. 1999).
5. *M. edulis* suffers less mortality from invertebrate predators, grading and socking (chapter 4; Miranda 2004).

Table 3.4. Co-existence mechanisms that have reasonable empirical support and relevance of their assumptions to co-existing *M. edulis* and *M. trossulus*.

Co-existence mechanism	**Spatial scale**	**Distinguishing assumptions used in mechanism development and their hypothesised relevance to co-existing *M. trossulus* and *M.edulis* as denoted by *=relevant**
Resource specialization[1] MacArthur and Levins 1967	Local/Regional	Differant species exploit distinct resources
Temporal specialization[1] Chesson 2000	Local	Species share same predators and/or resources* Differences arise depending on when species experience predation or exploit a resource
Spatial storage effects[1] Amarasekare 2003	Local Regional	SSE[2] and/or GDC[2]+dispersal establish source sink dynamics (+ CC/SN)* SSE and/or GDC establish source sink dynamics (+ CC/SN)*
Competition/ predator resistance[1] Chase et al. 2002	Local	Trade-off between predator resistance and competitive ability permits coexistence
Competition/ Longevity[1] Bonsall and Mangel 2004	Local	Trade-offs in longevity mitigate the effects of competitive ability
Competition/ colonisation (CC)[1,4] Tilman 1994 Kinzig et al. 1999 but see Levine & Rees 2002	Local/Regional	Trade-off between displacement ability against fecundity, recruitment, or dispersal ability* Competition is asymmetric with instaneous exclusion Disturbance from small (individual) to large scales* Patches reached by superior competitors cannot be colonised by inferior competitor
Successional niche (SN)[1] Pacala and Rees 1998 Bolker and Pacala 1999	Local	Trade-off between displacement ability and resource exploitation ability* Competitive asymmetry is imperfect, leading to slow exclusion* Inferior competitor can occupy any recently disturbed patch* Disturbance must occur over a small scale*
Intermediate Disturbance Hypothesis (IDH)[1] Shea et al. 2004	Local/Regional	Competition within community* Disturbance resets the successional process* The way in which disturbance regime scales with life-history is critical* Supported mechanisms: Relative non-linearity, storage effect (e.g.diapause), CC/SN*
Neutral theory[3] Hubbel 2001	Metacommunity	Life-history trade-offs act to equalise organism's fitness* Balance between speciation / stochastic extinction caused by random drifts in populations
Stochastic niche theory[1+3] Tilman 2004	Metacommunity	To establish, invaders must survive stochastic mortality while growing to maturity* Local diversity is limited by inhibitory effects of resource use by established species*

1. N: Niche based stabilizing co-existence mechanisms concentrate intra relative to interspecific competition, Intraspecific clustering is an emergent property and a potential barrier to hybridization in co-existing interbreeding species. Local/micro scale intraspecifc clustering of *M. edulis* and *M. trossulus* is occasionally observed (Miranda 2004; Bates and Innes 1995).
2. SSE: Spatial storage effect (SSE) and GDC: Growth density covariance (GDC).
3. Neutral / stochastic equalizing mechanisms of co-existence.
4. An analogous mechanism is the replacement CC hypothesis of Kisdi (2003) or varying degrees of replacement competition versus colonization abilities (Calcagno et al. 2006).

Chapter 4

Predator inducible defenses of co-existing *Mytilus edulis* and *M. trossulus*

4.1. Introduction

The optimal resource allocation strategy adopted by an individual organism is based on how it adapts to the threat of mortality (Williams 1966; Southwood 1977; Sibly and Calow 1986; Reznick et al. 1990; Boggs 1992; Stearns 1992, 2000). *Mytilus edulis* and *M. trossulus* are hybridizing blue mussel species with divergent resource allocation strategies (chapter 3), and co-exist throughout Atlantic Canada (Varvio et al. 1988; Koehn 1991; Bates and Innes 1995; Mallet and Carver 1995; Comesaña et al. 1999; Penney and Hart 1999; Hilbish et al. 2000; Rawson and Vanscoyoc 2001; Miranda 2004; Penney et al. 2006, 2007; Toro et al. 2002, 2004). Where *M. edulis* and *M. trossulus* coexist, *M. edulis* increases in frequency with decreasing size class (Comesaña et al. 1999; Miranda 2004; Toro et. al. 2004), implying differences in growth or survival. Differences between *M. edulis* and *M. trossulus* in allocation to growth or survival (in the absence of predation), together with the resultant intra- or inter-specific interactions, may not completely explain the often abrubt increase in the frequency of *M. edulis* among larger mussels (Mallet and Carver 1995; Comesaña et al. 1999; Miranda 2004, Toro et al. 2004; Penney et al. 2006, 2007; chapters 3, 5). One important source of mortality that has received less attention in mussel species, however, is the relative importance of "defensive" allocation and associated susceptibility to predation.

Diverting resources from absolute growth or reproduction to permanent defenses can incur fitness costs (Havel 1987; Harvell 1990). Consequently, where cues from predators are patchy in space and time, individual blue mussel's, similar to individuals from many aquatic taxa (Yoshioka 1982; Harvell 1990; Lively 1986; Appleton and Palmer 1988; Grosberg 1988; Palmer 1990; McCollum and Van Buskirk 1996; Trussell 1996), have evolved predator-inducible plastic defenses (Reimer and Tedengren 1996; Leonard et al. 1999; Reimer and Harms-Ringdahl 2001; Caro and Castilla 2004; Freeman

and Byers 2006; Freeman 2004, 2007). For example, after exposure to sea stars, crabs or predators with similar attack modes, a mussel can generally induce defenses in three ways. Firstly, individual mussels produce more and stronger byssal threads in high predation habitats, resulting in increased attachment strength (Seed and Suchanek 1992; Reimer and Tedengren 1996, 1999; Leonard et al. 1999; Reimer and Harms-Ringdahl 2001; Freeman 2007). Induced byssal thread production is an effective defense when predators try to dislodge or manipulate individual mussels from a clump (Reimer and Harms-Ringdahl 2001). Secondly, to counteract the crushing claws of crabs, a mussel can develop a thicker shell (Leonard et al. 1999; Smith and Jennings 2000; Reimer and Harms-Ringdahl 2001; Caro and Castilla 2004; Freeman and Byers 2006; Freeman 2007). Sea stars, on the other hand, pry apart the valves and can induce mussels to develop stronger adductor muscles and occasionally thicker shells (Hancock 1965; Reimer and Harms-Ringdahl 2001; Freeman 2007). The latter adaptation may be necessary where the strength of the adductor exceeds that of the shell. Thirdly, the presence of sea stars or crabs may induce reduced shell growth, which in combination with increased adductor, shell or attachment strength further enhances the induced defense (Leonard et al. 1999; Smith and Jennings 2000; Reimer and Harms-Ringdahl 2001; Caro and Castilla 2004). Alternatively, individual mussels may increase reproductive allocation when the benefits from reproduction outweigh the costs of the induced defense (Cote 1995; Riessen 1992; Reimer and Tedengren 1996, 1999).

In a field experiment described in chapter 3, *M. edulis* developed a thicker shell and a heavier adductor muscle than *M. trossulus*. Differences between the adductor muscle and shell thickness of *M. edulis* and *M. trossulus* could arise from a divergent response to cues from sea-stars, crabs or predators with similar attack modes (see above and Kitching et al. 1959; Hancock 1965; Seed 1976; Seed and Suchanek 1992; Reimer and Tedengren 1996, 1999; Leonard et al. 1999; Smith and Jennings 2000; Reimer and Harms-Ringdahl 2001; Caro and Castilla 2004; Freeman and Byers 2006; Freeman 2007). Predator inducible defenses may thus partially explain differences in shell

thickness between *M. edulis* and *M. trossulus* grown on or off bottom (Mallet and Carver 1995; Miranda 2004; Penney et al. 2007; chapter 3), although the relative abilities of co-existing *M. edulis* and *M. trossulus* to induce morphological defenses has never been quantified.

The objective of the experiment described in this chapter was to compare the defenses of *M. edulis* and *M. trossulus* induced by the presence of crabs or sea stars. To examine this, laboratory cultured *M. edulis* and *M. trossulus* were exposed to sea-stars and crabs and the subsequent allocation to absolute growth, adductor, shell thickness and attachment strength quantified, thereby providing information on the relative susceptibility of each mussel species to predation.

4.2. Material and Methods

4.2.1. General

Mussels (*M. edulis* and *M. trossulus*) were obtained from Salmonier Cove and Reach Run (NL) and identified with two allozymes (MPI and EST-D), 4 nuclear DNA markers (ME, ITS, MAL and PLIIa) and one mitochondrial DNA marker (COIII) (Miranda 2004). Pure-species families (5 families per species per site) were then produced in the laboratory in July 2001, see Miranda (2004). Mussels were initially reared for 18 months in the laboratory, where they were fed a standard hatchery rearing diet consisting of mixtures of live algae and raw seawater in a partial recirculation system. They were transferred in November 2001 to a mussel farm in Notre Dame Bay for 12 months, after which they were retrieved in November 2002, returned to the laboratory and reared in the laboratory as described above. Following this, all families for each species were pooled and reared in pearl nets for a further 9 months at Trinity, Trinity Bay, Newfoundland. Nine groups of 30 mussels of each genotype were subsequently sampled from the pearl nets in May 2004 and individually labelled using a Dremmel Tool™. After which they were acclimated in the laboratory for 2 months.

4.2.2. Byssal attachment, morphometric measurements, and dissection

Genotype groups were allocated to one of two separate compartments in 9 mesh trays each suspended in a 10 l aquarium from July-November 2004 (figure 4.1). Three aquaria were used for each of the crab (*Cancer irroratus*), sea star (*Asterias rubens*) or control (no predator) treatments, i.e. the treatments were triplicated. Mussels were conditioned in an aerated continuous flow-through system containing Shellfish Diet 1800 (Reed Mariculture) mixed with "raw" seawater at a daily ration of ~ 3% of the estimated total soft tissue biomass of the mussels in each tray. From a holding tank containing 20 crabs and 30 sea stars; two crabs and three sea stars were rotated into and out of each of the appropriate treatment aquarium every 3 days. At any given time each treatment aquarium contained approximately the same predator biomass. Mussels were acclimated in the trays for 2 weeks before predators were introduced. Observations of separate groups of mussels reared in identical aquariums with the same conditioning diet suggested that mussels were spawned out by September 2004 and were actively recovering from spawning up until the end of the experiment in late November 2004.

Attachment force (g), measured with modified forceps and a force gauge, was determined for each mussel in September (40 days) and late November (122 days). The forceps, connected to the force gauge with inflexible braided fishing line (100lb breaking strain), were modified such that they would clamp or hook around the mussel without applying undue pressure to it or altering its position. Once the forceps were attached to the mussel the force gauge was carefully elevated until the mussel detached from the substrate. Only individuals attached directly to the mesh trays were included in the analysis, mussels attached to each other being excluded. Shell length (anterior-posterior axis), height (dorso-ventral axis), width (lateral axis) were measured (± 0.1mm) with vernier calipers at the beginning (August 2004) and end (November 2004 or 122 days) of the predation experiment. After termination of the experiment in late November 2004, the remaining individuals were stored at –20°C. The adductor muscle, remaining soft body

tissues and shell were subsequently removed and dried separately to constant weight at 80°C, then weighed to the nearest 0.0001g.

4.2.3. Rate of attack by sea stars

Sea star attack rate on 60 *M. edulis* and 60 *M. trossulus*, equally divided into two compartments of duplicate mesh trays suspended in two 10 litre aquaria, was recorded during July- August 2004 for 60 days. Sea stars sampled from a holding tank of 30 were rotated into and out of the aquaria every 3-5 days. To prevent position effects, the trays were randomly re-orientated within the aquaria every three days. Mussels were conditioned as described above and observed at approximately 15-minute intervals from 9-5pm Monday to Friday for 60 days. A sea star attack was recorded when a sea star had settled under a mussel and begun to evaginate its stomach through the mesh of the tray. At this point the sea star was gently removed from the bottom or sides of the mesh tray and placed at the bottom of the aquarium.

4.2.4. Statistical analyses

All analyses were conducted in the R-statistical environment version 2.5.0 (Williams et al. 2000; Dalgaard 2002, Faraway 2002, 2004). Statistical testing of growth rates, calculated from marked individuals as the difference between final and initial shell length, was carried out by two-way ANOVA with genotype and treatment as fixed effects and aquaria nested within both treatment and genotype X treatment as random effects. Only individuals that could be accurately identified by their individually assigned labels were included in the analyses. Individual labeling permitted more accurate growth estimates while at the same time distinguishing the two species. Data for byssal attachment force, adductor mass and shell deposition were analyzed by two-way ANCOVA of these dependent variables, with genotype and treatment as fixed effects and aquaria nested within both treatment and genotype X treatment as random effects. Individuals that were not attached to mesh trays were excluded from the analyses. All

pair-wise contrasts between treatments within each genotype or between genotypes within each treatment were adjusted by the Bonferroni method (Sokal and Rohlf 1995). All tests complied with the assumptions of normality and homogenous distribution of residuals required in GLM models. Additionally, sea star attack rates between species were contrasted using log likelihood ratio tests of independence and goodness of fit, with Yates corrections.

4.3. Results

4.3.1. Absolute growth

Both predator treatments reduced growth in *M. trossulus* (control treatment> crab treatment> sea star treatment) while only the sea star treatment reduced growth of *M. edulis* (control treatment= crab treatment, > sea star treatment) (Table 4.1, Figure 4.2). Shell length growth in both the control and crab treatments was significantly greater in *M. edulis* than in *M. trossulus*. In the sea-star treatment, however, shell length growth was not significantly different between the species (Figure 4.2).

4.3.2. Attachment force

After 40 days in September 2004, predator exposure resulted in significantly increased attachment force in *M. trossulus* and *M. edulis* in both the crab and sea star treatments compared with the control treatment (Figure 4.3, Table 4.2). *M. trossulus* required 540% more force to detach from the substrate when exposed to crabs than did the control group, and 249% more force in the case of exposure to sea stars. The corresponding value in *M. edulis* for both crabs and sea stars was ~700% (Figure 4.3). Predator exposure for a further 82 days up to late November 2004 (during recovery from spawning) again resulted in significantly increased attachment in *M. trossulus* and *M. edulis*, although predator treatments were also significantly different (for both species crab treatment > sea star treatment > control treatment) (Table 4.2, Figure 4.4). Relative

to its control treatment, *M. trossulus* required 450% more force respectively to detach from the substrate when exposed to crabs and 189% more force following exposure to sea stars. *M. edulis* required 903% more force than the control to detach from the substrate in the crab treatment and 571% in the sea star treatment. The attachment force of *M. edulis*, compared with *M. trossulus* during spawning recovery was only significantly greater in the sea star treatment, significantly weaker in the control and not significantly different in the crab treatment (Figure 4.4, Table 4.2).

4.3.3. Shell and adductor mass

Exposure to both predator species resulted in a significant increase in shell weight for *M. edulis* (crab treatment > sea star treatment > control treatment) but not *M. trossulus* (crab = sea star = control) (Figure 4.5, Table 4.3). For example, *M. edulis* shell mass, standardized to a 30 mm mussel, increased from the control value by approximately 34% or 15% in the crab and sea star treatments respectively. Furthermore, exposure of mussels to sea stars resulted in a significant and large increase in adductor mass only in *M. edulis* (sea star treatment > [crab treatment= control treatment]) (Figure 4.6, Table 4.4).

4.3.4. Sea star attack rate

During an observation period of 60 days, *M. trossulus* was attacked significantly more frequently than *M. edulis* (*M. trossulus*: 75 of 101 recorded attacks; *M. edulis*: 26 of 101 recorded attacks: G-test, df = 1 G= 49.25, $P < 0.0001$).

4.4. Discussion

Adaptive phenotypic plasticity is useful to organisms because it allows the temporary adoption of morphologies and behaviors that might otherwise be prohibitively expensive, in terms of fitness costs, to maintain continually (Harvell 1990). Inducible defenses are among the most well researched and taxonomically widespread examples of phenotypic plasticity. They include behavioral, morphological and physiological changes that are generally attributed to 'predator cues' which in turn increase resistance to predatory attacks (Harvell 1990). The predator inducible defenses observed here, to resist the crushing claws of crabs or increase the effort required for sea stars to pry open the shell valves, are generally consistent with other studies on blue mussels (Hancock 1965; Hughes and Seed 1981; Reimer and Tedengren 1996; Leonard et al.1999; Reimer and Harms-Ringhdahl 2001; Caro and Castilla 2004; Freeman and Byers 2006; Freeman 2004, 2007). None of these studies, however, included a genetic component.

An induced response in a prey organism can arise after exposure to "infochemicals" either from the predator itself (enemy-avoidance kairomones; Ruther et al. 2002) or from damaged and ingested prey conspecifics (alarm pheromones; Smith 1992; Stabell et al. 2003), or simply following non-lethal physical contact with the predator (Leonard et al. 1999). The induced phenotypes observed in the current experiment were likely attributable to a combination of "enemy avoidance kairomones " or from physical contact with the predators on occasions where crabs or sea stars had moved onto the underside of the mesh tray supporting the mussels in each aquarium. Shell thickening provides increased protection from the crushing action of crab claws and has commonly been reported in bivalves and gastropods (Leonard et al. 1999 and citations therein). Following exposure to crabs, *M. edulis* developed a thicker shell while *M. trossulus* did not. In response to sea star exposure *M. edulis* developed a greatly enlarged adductor muscle, which is in agreement with other studies (Reimer and Tedengren 1996; Reimer and Harms-Ringhdahl 2001), but *M. trossulus* did not. The observed predator induced increase in byssal attachment in both mussel species is likely

an efficient defense against both sea stars and crabs, since the mussel becomes more difficult to remove from the substrate (Auster 1986; Reimer and Tedengren 1997; Reimer and Harms-Ringhdahl 2001). The attachment of *M. edulis* in the predator treatments was much stronger than *M. trossulus* immediately after spawning, and generally remained stronger during recovery from spawning. The allometric increase in variables such as adductor weight and shell thickness, important components of predator inducible defenses, is associated with depressed growth in blue mussels during exposure to invertebrate predators and likely reflects a trade-off between defensive traits and absolute growth of shell (Leonard et al. 1999; Caro and Castilla 2004; Freeman and Byers 2006; Freeman 2004, 2007). The observed reductions in the shell-length growth of *M. edulis* in the presence of sea stars and *M. trossulus* in the presence of sea stars or crabs supports this conclusion. However, since shell-length growth in *M. edulis* was not influenced by the presence of crabs, the increase in shell thickness and attachment strength in *M. edulis* likely reflects diversion of energy from reproduction rather than absolute growth (Auster 1986; Lively 1986; Zardi et al. 2007). Thus *M. edulis* can both defend against the threat of crab predation through induced defenses while at the same time growing towards a potential size refuge from crab predation (see Seed and Suchanek 1992).

The adaptive significance of predator inducible defenses is well documented (Leonard et al. 1999; Smith and Jennings 2000; Caro and Castilla 2004; Freeman 2007). In the present experiment, the predator-induced increases in shell and adductor mass and in attachment strength in both species were consistent with the findings of Leonard et al. (1999), Caro and Castilla (2004), and Freeman (2007) for mytilids. Other studies have also demonstrated a clear relationship between shell thickness or mass and resistance to breakage (Mallet and Carver 1995; Miranda 2004; Penney et al. 2007). Data from this study suggest that *M. trossulus* is more susceptible than *M. edulis* to predation by sea stars and crabs. The preference of sea stars for *M. trossulus* during the reproductive period supports this conclusion. Periods of selective predation on *M. trossulus* could therefore explain the decreasing frequency of *M. trossulus* with increasing size (Comesaña et al. 1999; Toro et. al. 2004; Miranda 2004), especially where the shell-

length growth of *M. edulis* is depressed during exposure to sea stars or at relatively high population densities of mussels (chapter 5). This in turn would increase the likelihood of *M. edulis* displacing *M. trossulus*, resulting in the dominance of *M. edulis* in patches that have not experienced indiscriminate disturbance. Differences between the two species in key defensive traits (increases in shell and/or adductor muscle mass) were only apparent in the presence of sea stars or crabs. Consequently, observed differences in shell weight or thickness (Mallet and Carver 1995; Miranda 2004; Penney et al. 2007; chapter 3) or adductor muscle weight (chapter 3) are at least partly attributable to phenotypic plasticity induced by non-lethal exposure to predators.

The lesser degree to which *M. trossulus* induces defenses in the presence of predators supports the argument that *M. trossulus* prioritizes allocation of resources to reproductive rather than somatic functions relative to *M. edulis* earlier in life (chapter 3). Relative to *M. edulis*, increased allocation to reproduction, in turn, enhances *M. trossulus* ability to recolonize disturbed patches, thereby increasing its resilience rather than its resistance to disturbance (Southwood 1977; chapters 1, 3). Differences between *M. edulis* and *M. trossulus* in resilience or resistance to disturbance arising from divergent resource allocation patterns (see chapter 3) could theoretically reinforce stabilizing mechanisms pertaining to their coexistence (Chesson 2000). Furthermore, because of the differences between *M. edulis* and *M. trossulus* in the ability to induce defenses, the interpretation of studies on inducible defenses where the two species co-occur but are not uniquely identified could be confounded. Freeman and Byers (2006), for example, suggested that there has been rapid evolution of an inducible morphological response in *Mytilus edulis* to the crab *Hemigrapsus sanguineus* within 15 years of its introduction to southern New England (USA) (but see Rawson et al. 2007). Freeman and Byers (2006) originally assumed, though, that the study species was exclusively *M. edulis*, whereas in fact it was a mixture of *M. edulis* and *M. trossulus*. Following the discovery that both species may actually have been present in Freeman and Byers (2006) study (Rawson et al. 2007), tissues from the experimental mussels were subsequently genotyped. The number of *M.*

trossulus sampled in Freeman and Byers (2006), however, was small relative to *M. edulis* so that the results were similar whether or not *M. trossulus* was included in the analyses (Freeman and Byers 2007). In contrast, the present experiment was based on highly controlled conditions in the laboratory using much larger sample sizes of mussels of known species composition.

An important caveat of the current experiment was that growth rates were relatively low compared with field experiments over a similar time period (chapters 3, 5), implying that despite the feeding regime mussels were food limited in the laboratory. Due to bio-fouling of the supply pipes, raw seawater pumped to the experimental aquaria contained relatively little phytoplankton, while the mixed species algal concentrate used to feed the mussels was likely an imperfect substitute for algae in the wild. Given more food *M. trossulus* may have induced defenses once the requirements of its increased investment to reproduction had been met. *M. trossulus* was certainly able to recognize non-lethal cues from sea stars or crabs and increase attachment strength accordingly. Presumably the reproductive costs in this instance were lower than the costs of the defense associated with the predator (Harvell 1990). Nevertheless, it is likely that with a better food supply *M. edulis* would still prioritize defensive allocation to a greater degree than *M. trossulus*. Evidence for this comes from a field experiment (chapter 3) at a location where invertebrate predator cues were present, in which *M. edulis* developed thicker shells and larger adductor muscles than *M. trossulus* i.e. consistent with the laboratory experiment. The current experiment, then, most closely simulates situations in which predator density is high and/ or mussels are food limited.

In conclusion, the current experiment supports growing evidence that predation risk alone (i.e. non-lethal effects) can drive trophic interactions and play an important role in shaping community structure and dynamics (Turner et al. 2000; Werner and Peacor 2003 and citations therein). For example, predators may induce adaptive changes in the phenotype of their prey, thereby influencing the interactions between prey and their

predators. The experiment also demonstrates that co-existing sibling species may differ in their response to predators such that one species (*M. edulis*) is more resistant to predators than the other species (*M. trossulus*), which is potentially more resilient (chapter 3). These types of rapidly induced defenses and the resultant predator-prey interactions, together with differences in species resistance or resilience to predation, in turn impact the temporal and spatial distribution of the species and their subsequent potential to coexist through successional dynamics (chapters 3, 6).

4.5. References

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Figure 4.1. Example of a mesh tray used for predation experiments. One tray was suspended in each of nine 10 l aquaria (3 aquaria for each treatment: crab, sea star, control). Each tray was divided into two compartments, one containing 30 *M. edulis* (E), the other 30 *M. trossulus* (T).

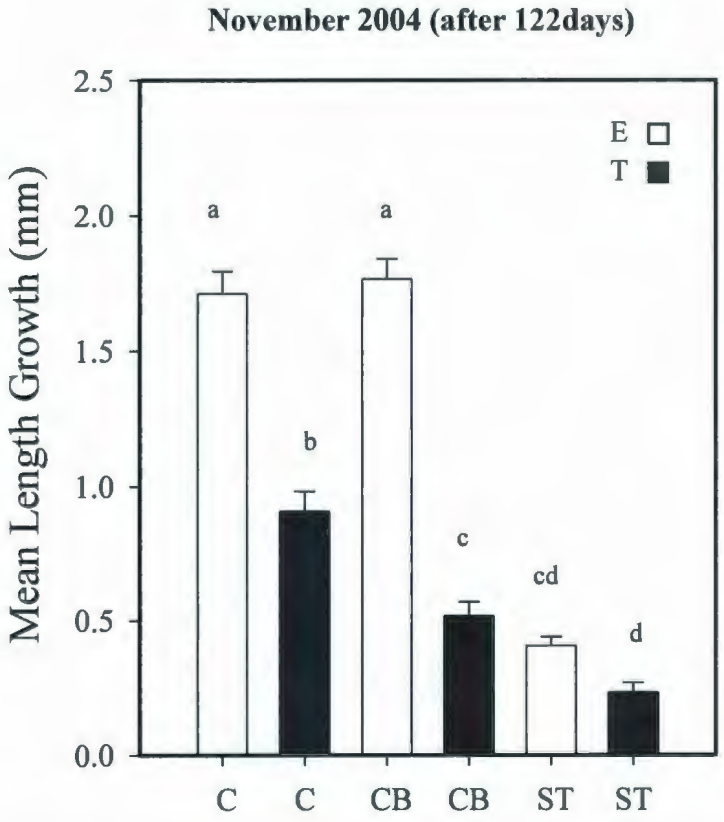

Figure 4.2. Mean shell length growth (mm) from August-November 2004 (122 days) in *M. edulis* (E) and *M. trossulus* (T) in the control (C), crab (CB) and sea star treatments (ST). Error bars represent ± 1 standard error. Genotype and treatment combinations with the same letter are not significantly different from each other.

Table 4.1. Two-Way nested ANOVA (Unbalanced, Type III Sum Sq., General Linear Model) for shell length growth (mm) from August to November 2004 (122 days).

Growth					
	Df	Sum Sq	Mean Sq	F value	P
Treatment	2	86.99	43.50	191.05	***
Species	1	54.75	54.75	153.08	***
Treatment x Species	2	19.50	9.75	27.25	***
Aquaria (Treatment)	6	1.37	0.23	0.76	
Aquaria (Species x Treatment)	6	2.15	0.36	1.20	
Residuals	402	120.06	0.30		

Main effects
Genotype: *M. edulis*, *M. trossulu.*
Treatment: crab, sea star, control
Nested factor
Aquaria nested within treatment, aquaria nested within species X treatment
* P<0.05, ** P<0.01, *** P<0.001, ns non-significant

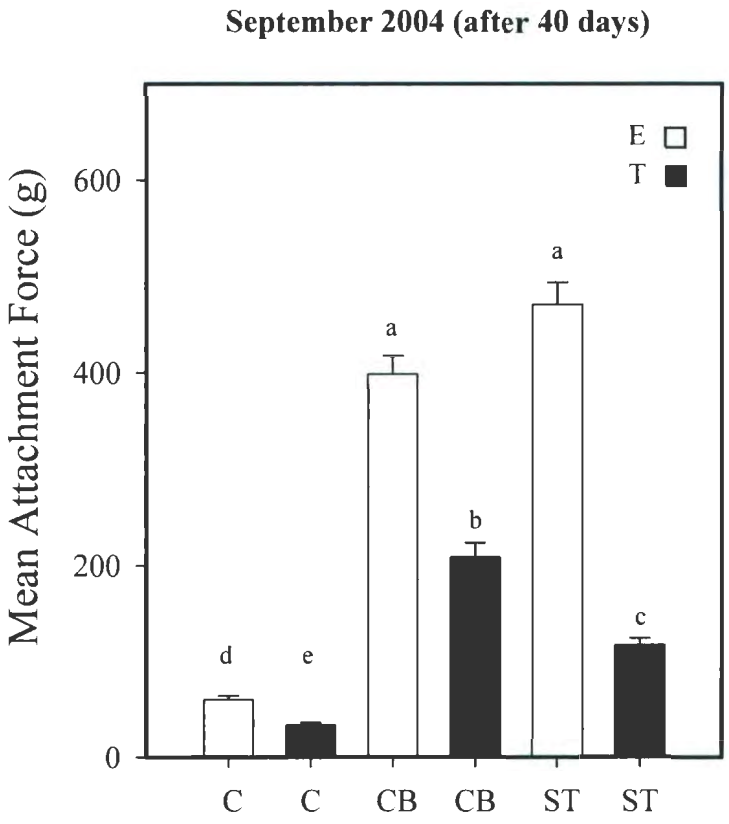

Figure 4.3. Mean attachment force (g) of *M. edulis* (E) and *M. trossulus* (T), corrected for shell length (21mm), after 40 days in the control (C), crab (CB) or sea star treatments (St). Error bars represent ± 1 standard error. Genotype and treatment combinations with the same letter are not significantly different from each other.

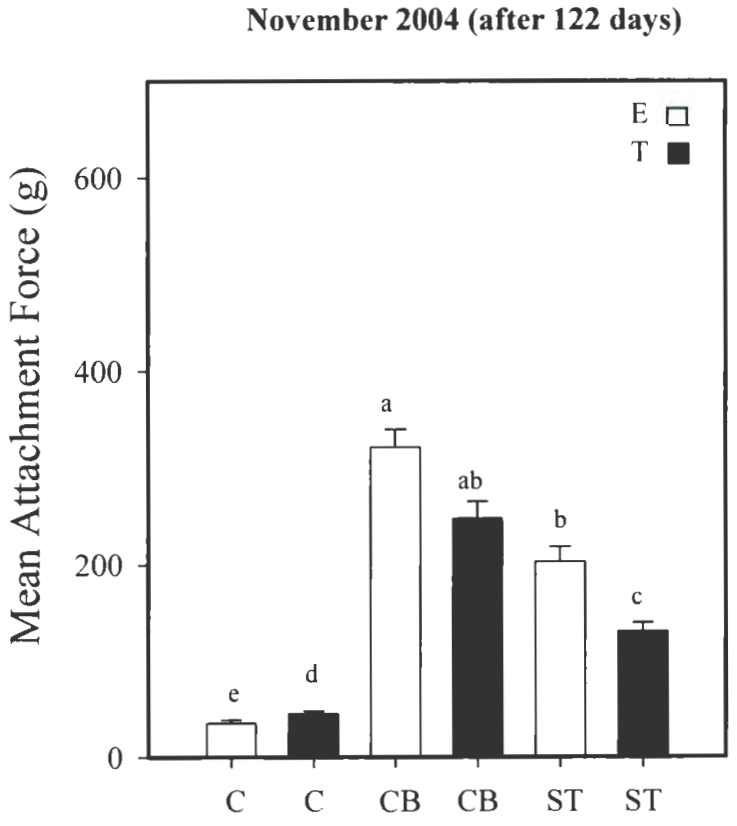

Figure 4.4. Mean attachment force (g) of *M. edulis* (E) and *M. trossulus*, corrected for shell length (21mm), after 122 days (T) in the control (C), crab (CB) or sea star treatments (St). Error bars represent ± 1 standard error. Genotype and treatment combinations with the same letter are not significantly different from each other.

Table 4.2. Two-Way nested ANCOVA (Unbalanced, Type III Sum Sq., General Linear Model) for attachment force (ln x+1 (g)).

ln x+1 Attachment during spawning (g) (September (40 days))					
	Df	Sum Sq	Mean Sq	F value	P
Length (ln x+1)	1	19.20	19.20	71.14	***
Treatment	2	52.92	26.46	98.06	***
Species	1	14.95	14.95	64.59	***
Treatment x Species	2	2.42	1.21	5.22	*
Aquaria (Treatment)	6	1.62	0.27	5.85	*
Aquaria (Species x Treatment)	6	1.39	0.23	5.02	*
Residuals	403	18.58	0.05		

ln x+1 Attachment post spawning (g) (November (122 days))					
	Df	Sum Sq	Mean Sq	F value	P
Length (ln x+1)	1	16.42	16.42	261.99	***
Treatment	2	46.87	23.44	373.97	***
Species	1	0.23	0.23	3.07	ns
Treatment x Species	2	2.14	1.07	14.33	***
Aquaria (Treatment)	6	0.38	0.06	1.06	ns
Aquaria (Species x Treatment)	6	0.45	0.07	1.27	ns
Residuals	354	20.91	0.06		

Main effects
Genotype: *M. edulis*, *M. trossulus*
Treatment: crab, sea star, control
Nested factor
Aquaria nested within treatment, aquaria nested within species X treatment
Covariate
Shell length (ln x+1)
* P<0.05, ** P<0.01, *** P<0.001, ns non-significant

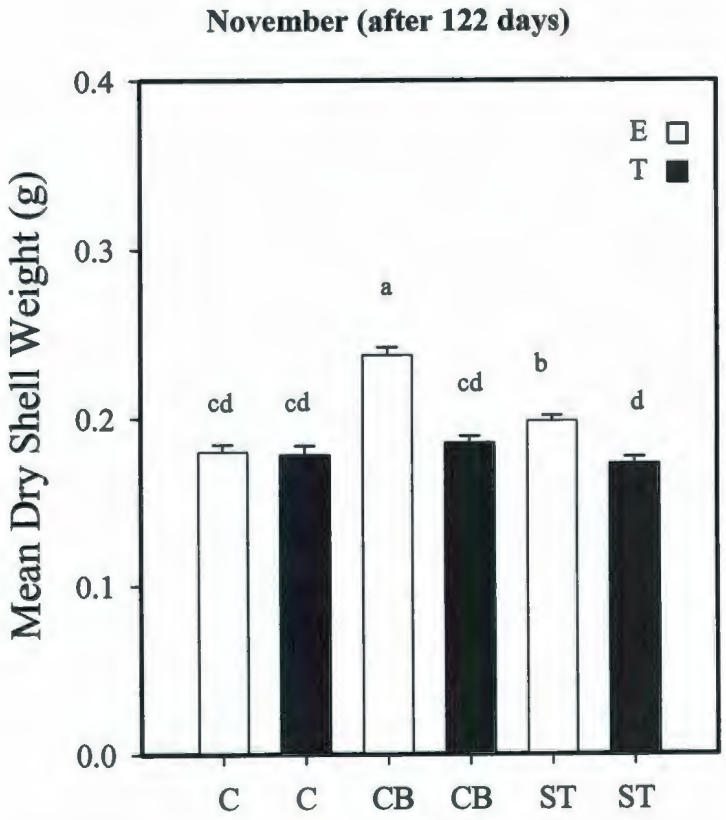

Figure 4.5. Mean shell mass (g) of *M. edulis* (E) and *M. trossulus*, corrected for shell surface area (3.2cm^2), after 122 days (T) in the control (C), crab (CB) or sea star treatments (St). Error bars represent ± 1 standard error. Genotype and treatment combinations with the same letter are not significantly different from each other.

Table 4.3. Two -Way nested ANCOVA (Unbalanced, Type III Sum Sq., General Linear Model) for shell mass (ln x+1 (g)) after 122 days.

ln x+1 Shell weight (g)					
	Df	Sum Sq	Mean Sq	F value	P
SA (ln x+1)	1	23.406	23.406	11417.37	***
Treatment	2	0.400	0.200	97.44	***
Species	1	0.589	0.589	91.78	***
Treatment x Species	2	0.228	0.114	17.78	***
Aquaria (Treatment)	6	0.012	0.002	0.63	ns
Aquaria (Species x Treatment)	6	0.039	0.006	1.98	ns
Residuals	442	1.434	0.003		

Main effects
Genotype: *M. edulis*, *M. trossulus*
Treatment: crab, sea star, control
Nested factor
Aquaria nested within treatment, aquaria nested within species X treatment
Covariate
Shell Surface area "SA" (ln x+1)
* P<0.05, ** P<0.01, *** P<0.001, ns non-significant

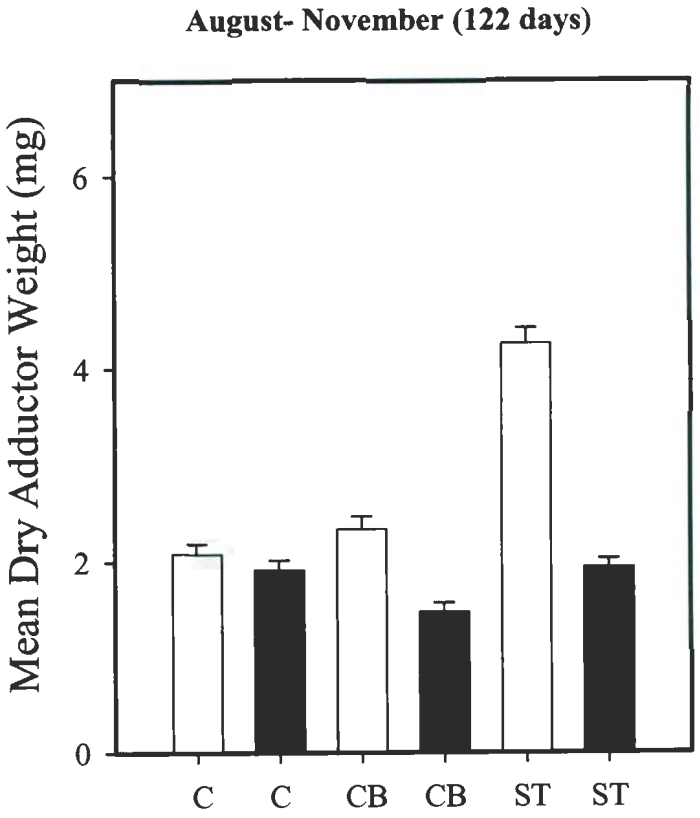

Figure 4.6. Mean adductor mass (mg) of *M. edulis* (E) and *M. trossulus*, corrected for shell surface area (3.2cm^2), after 122 days (T) in the control (C), crab (CB) or sea star treatments (St). Error bars represent ± 1 standard error. Genotype and treatment combinations with the same letter are not significantly different from each other.

Table 4.4. Two-Way nested ANCOVA (Unbalanced, Type III Sum Sq., General Linear Model) for adductor muscle mass (ln x+1 (g)) after 122 days.

ln x+1 Adductor weight (g)					
	Df	Sum Sq	Mean Sq	F value	P
SA (ln x+1)	1	17.421	17.421	155.18	***
Treatment	2	4.405	2.202	19.62	***
Species	1	4.173	4.173	86.60	***
Treatment x Species	2	1.338	0.669	13.88	**
Aquaria (Treatment)	6	0.674	0.112	7.05	*
Aquaria (Species x Treatment)	6	0.289	0.048	3.03	ns
Residuals	442	7.040	0.016		

Main effects
Genotype: *M. edulis*, *M. trossulus*
Treatment: Crab, sea star, control
Nested factor
Aquaria nested within treatment, aquaria nested within species X treatment
Covariate
Shell surface area "SA" (ln x+1)
* P<0.05, ** P<0.01, *** P<0.001

Chapter 5

Intra- and inter-specific interactions among coexisting blue mussel species (*M. edulis- M. trossulus)* in response to crowding

5.1. Introduction

Sessile suspension-feeding invertebrates are well suited to the study of density-dependent interactions because, as with plant species, they form fairly stable assemblages through time (Weinberg 1998) and are often limited by space and nutrients (Hughes and Griffiths 1988). Crowding may be associated with decreased growth or survival in sessile organisms (Yoda et al. 1963; Harper 1977; Westoby 1984; White 1985; Seed and Suchanek 1992; Guinez et al. 2005; but see Bertness et al. 1998). Moreover, population density is a key factor influencing body-shape, together with ontogeny (Dickie et al. 1984; Stirling and Okumu 1994; Karayücel and Karayücel 2000) and environmental factors such as predator exposure (Reimer and Tedengren et al. 1996; Leonard et al. 1999; Reimer and Harms-Ringdahl 2001), wave exposure and tidal height (Seed 1973; Raubenheimer and Cook 1990; Akester and Martel 2000; Beadman et al 2003). This may result in, for example, more elongated shells in mussels (Coe 1946; Lent 1967; Seed 1968, 1973; Richardson and Seed 1990), oysters (Tanita and Kikuchi 1957) and clams (Ohba 1956; Cigarria and Fernanadez 1998), and even extreme deformities (Bertness and Grosholz 1985).

One of the most spectacular effects of high population density on individual body-form in benthic suspension-feeders can be seen within the hummocks formed by barnacle populations. Rather than reducing the fitness of crowded individual barnacles, density may actually increase it by buffering individuals from heat or desiccation stress, thereby boosting reproductive output (Bertness et al. 1998). The effect of density on *Mytilus* species has been particularly well documented. In general, high population density acts on the size and shape of *Mytilus* individuals primarily through food regulation, physical

interference, or their interaction (Wildish and Kristmanson 1984; Frechette and Bourget 1985a, b; Alunno-Bruscia et al. 2000, 2001). Because blue mussels may clump in a 3 dimensional matrix, overcrowding and the associated decreased access to space or food (if limited) can result in suppressed growth or increased mortality of underlying individuals (Griffiths and Hockey 1987; McGrorty et al. 1990; Richardson and Seed 1990). Such interactions are most intense in relatively stable assemblages of rapidly growing mussels (Griffiths and Hockey 1987). Conversely, in slow growing mussels that take longer to reach a size refuge from predation, particularly those with a small terminal body size, the population may be controlled more by predation than by density-dependent interactions (Griffiths and Hockey 1987).

Body size is a key factor in determining the outcome of competitive interactions in *Mytilus* species (Harger 1968; Seed and Suchanek 1992). Smaller individuals for example suffer disproportionately more than larger individuals from resource shortages or agonistic interactions (Schmitt et al. 1987; but see Baardvik and Jobling, 1990; Weiner 1990; Adams et al. 2000). Furthermore, because large individuals are less susceptible to crab predation, they may "pre-emptively" inhibit the establishment of mussels through succession, either by direct ingestion of plantigrades or by occupying space that would otherwise be colonized (Seed and Suchanek 1992). Despite accounts of density-dependent intra-specific interactions in *Mytilus* species, density-dependent inter-specific interactions have not been well documented. Furthermore, the effect of density on intra-specific interactions has not been directly measured among co-existing *Mytilus* species.

The objective of the current study was to determine how co-existing *Mytilus* species respond to crowding in terms of growth, survival and shell morphology. *Mytilus edulis* and *M. trossulus* coexist and interbreed throughout Atlantic Canada. More recently *M. trossulus* from the Baltic has been introduced into Scotland, where it now co-exists with *M. edulis* and *M. galloprovincialis* (Brown, J., *pers. comm.*). *M. edulis* and *M. trossulus* are fairly typical of co-existing *Mytilus* species in that one species, *M. edulis* in this

hybrid zone, occurs at a greater frequency with increasing size class (Comesaña et al. 1999; Miranda 2004; Toro et. al. 2004). Because *M. edulis*, relative to *M. trossulus*, prioritizes allocation to somatic growth and because faster growing mussels are more susceptible to negative intra-specific interactions, it was hypothesized that the shell growth, morphology, or survival of *M. edulis* would be more strongly affected by increasing density. This in turn could help to explain the wide range of shell growth rates of *M. edulis* relative to *M. trossulus* (Mallet and Carver 1995; Penney et al. 2002; Miranda 2004; Mallet and Carver 1995; Penney et al. 2006; Chapter 2). Furthermore, it was hypothesized that where the two species co-occur *M. edulis* would grow faster and subsequently occupy a greater proportion of the available space than *M. trossulus*, such that *M. trossulus's* growth would be suppressed.

5.2. Material and methods

5.2.1. Experimental design

By following laboratory-produced cohorts of each species it was possible to control for the effects of age (Bayne et al. 1983; Kautsky 1982; Sprung 1983; Thompson 1984; Rodhouse et al. 1985) and position (Okamura 1986), both of which are potentially confounding factors when studying the relationship between growth and density. After identification of *M. edulis* and *M. trossulus* parents obtained from Salmonier Cove, Drac Bay and Reach Run (NL) with two allozymes (MPI and EST-D), 4 nuclear DNA markers (ME, ITS, MAL and PLIIa) and one mitochondrial DNA marker (COIII) (Miranda 2004), *M. edulis* and *M. trossulus* families (n=5 per species) were produced in the laboratory in July 2002 (Miranda 2004). Prior to the experiment, all families within each genotype were pooled and reared in identical conditions in the laboratory (August 2002- November 2003), after which they were over-wintered in pearl nets suspended in the water column at Trinity (Trinity Bay, Newfoundland). Mussels reared in the laboratory were fed a

standard hatchery rearing diet consisting of mixtures of live algae and raw seawater in a partial recirculation system.

Long Harbour, Placentia Bay, was selected as the experimental site because previous experiments had demonstrated that growth rates were very high for mussels deployed there (Miranda 2004; Chapter 3), thereby reducing the time needed to detect potentially density-dependent interactions. Samples of 702 *M. edulis* (shell length 20.7mm ± 3.6SD) and 702 *M. trossulus* (19mm ± 3SD) were then assigned to 500cm^3 cages at 5 densities (6, 14, 30, 46, 60 mussels per cage), each density being triplicated (Figures 5.1, 5.2, Table 5.1). Of the 1404 mussels assigned to the cages, 840 (420 of each species) were individually labeled with a Dremmel Tool™ (Table 5.1). Cages were housed in five strings of three pearl nets and deployed at Long Harbour in June 2004. Pearl nets were cleaned of bio-fouling and re-arranged at monthly intervals to minimize spatial effects.

Measurements of the shell dimensions of marked individuals were made before deployment at Long Harbour (June 2004), immediately after spawning (September 2004) and in December 2004. For each marked individual, shell length (anterior-posterior axis), height (dorso-ventral axis), and width (lateral axis) was measured to the nearest 0.1mm with vernier calipers. For shell shape contrasts, ratios of shell length: height or shell height: width were derived for marked individuals at the end of the experiment. Survival was also estimated from the number of marked individuals remaining alive at the end of the experiment compared with the number deployed at the beginning of the experiment.

5.2.2. Statistical analyses

A multivariate analysis was not adopted because it is not yet possible to combine binary (survival data) and continuous data (shell growth and shell shape) (see Ernande et al. 2004). Consequently, to reduce the probability of a Type 1 error when carrying out multiple comparisons from the same data-set, significance levels for all parameters used in the ANCOVAs were computed with the Bonferroni correction (Sokal and Rohlf 1995). The 5 densities were treated as a continuous explanatory variable in a regression based analyses rather than a fixed factor in an ANOVA (Sokal and Rohlf 1995; Dalgaard 2002). The transition from an ANOVA to ANCOVA in this instance is a form of model simplification that accommodates multiple measurements of y (the response variable) for each value of x (the independent variable). Accordingly, the error associated with the "deviance" from regression was computed before the minimally adequate statistical contrasts were made. In all instances the variation attributable to the "deviation" from the regression term was not significant and was removed from the model using the step function in R. Shell shape ratios were also arcsine-square root transformed. To test for differences among densities in shell growth, shell length: height and shell height: width, each of these dependent variables was regressed against density. First, regression coefficients among groups (*M. edulis*, *M. trossulus*, *M. edulis* or *M. trossulus* at a 1:1 mixture) were compared by ANCOVA. Bonferroni adjusted pair-wise contrasts were computed if regression coefficients differed among groups. The Johnson-Neyman procedure was used for pair-wise contrasts when regression slopes were not homogeneous, thereby allowing estimates of the region of x (density) where the y values (response variable) were non-significant (White 2003). Only marked individuals that could be identified were included in the analyses. The advantage of including tagged individuals was that growth could be determined with great accuracy. An ANOVA of differences among final average length between tagged and untagged individuals, undertaken to determine the effect of marking on growth, confirmed that final shell length, length: height, and height: width did not differ between tagged and untagged

individuals. The effect of density and group on the proportion of dead individuals at the end of the experiment was analyzed by Analysis of Deviance (Crawley 2003) using a binomial error structure to accommodate the proportion data (Generalized Linear Model).

5.3. Results

5.3.1. Shell length growth

The growth of both *M. edulis* groups declined at a greater rate than both *M. trossulus* groups with increasing mussel density (Figure 5.3, Table 5.2). Further the only case where density had no significant effect on growth was when *M. trossulus* was mixed with *M. edulis* after 90 days (Linear regression: $F_{1,186}$= 3.38, P= 0.07). Additionally, because slopes were homogenous, shell length growth after 90 or 150 days was significantly greater by approximately 2.5 mm (12% of initial shell length) for *M. edulis* mixed at a 1:1 ratio with *M. trossulus* compared with the pure *M. edulis* group at all densities (Figure 5.3, Table 5.2). In contrast, the shell growth rate of *M. trossulus* in the presence of *M. edulis* was significantly lower than that of *M. trossulus* in the absence of *M. edulis* at the highest densities after 90 days or 150 days of growth, respectively (Figure 5.3, Table 5.2). Moreover, when each species was maintained in the absence of the other there was no significant difference between species in shell length growth at the highest density, both at 90 and 150 days (Figure 5.3, Table 5.2). The growth of *M. edulis* mixed with *M. trossulus*, however, was significantly greater than that of either *M. trossulus* group at all densities (Figure 5.3, Table 5.2).

5.3.2. Shell shape

As the ratio of shell length: height increases or the ratio of height: width decreases, the shell effectively becomes more elongated and less rounded. Generally the ratio of shell length: height increased, or height: width decreased, with increased mussel

density (Figure 5.4). Density, though, had no significant effect on the ratio of shell height: width when *M. trossulus* was grown with *M. edulis* (Linear regression: $F_{1,160}$= 0.002, P= 0.96). Because there was no significant difference in shell shape between treatments (presence or absence of congenerics) for either *M. edulis* or *M. trossulus*, groups for each genotype were analyzed as *M. edulis* or *M. trossulus* (species) only (Table 5.3). Overall at all densities, the ratio of shell length: height was greater, or height: width lower, in *M. edulis* than *M. trossulus* (Figure 5.4, Table 5.3).

5.3.3. Mortality

Density did not explain a significant amount of the variation in mortality rate within any of the groups (Figure 5.5, Table 5.4a). According to pair-wise contrasts among groups at each density level (Table 5.4b), mortality was only significantly different between *M. edulis* and *M. trossulus* in the mixed group at a density of 30 mussels per cage (Chisq= 9.2, df= 1, P= 0.0024, Figure 5.5).

5.4. Discussion

With the exception of *M. trossulus* mixed with *M. edulis* following 90 days of growth, density significantly influenced the growth of pure *M. edulis*, *M. trossulus,* and the growth of either *M. edulis* or *M. trossulus* mixed at a 1:1 ratio with the other species. The observed reductions in growth with increasing density may be attributable to food and/ or space limitation (Peterson 1982; Wildish and Kristmanson 1984; Fréchette and Bourget 1985a,b; Alunno-Bruscia et al. 2001) and/ or physical interference arising from asymmetric competition (Harger 1968; Seed 1968; Fréchette and Desplaud 1999). The latter occurs when larger individuals obtain a disproportionate share of the limiting resource, thereby decreasing the fitness of smaller individuals (see Weiner 1990; Fréchette and Desplaud 1999 for examples in plants and mussels respectively). This interpretation is complicated by important differences among pure *M. edulis*, *M.*

trossulus, and *M. trossulus* or *M. edulis* mixed at a 1:1 ratio with the other species. For example, reductions in density had a much stronger positive effect on shell length growth in pure *M. edulis* or *M. edulis* mixed with *M. trossulus* than in either *M. trossulus* group. At all densities *M. edulis* grew faster in the presence of *M. trossulus* than it did in the absence of the latter. In contrast, at the highest densities *M. trossulus* grew faster in the absence of *M. edulis* than it did in the mixed group. Density also influenced shell shape, albeit weakly, which is consistent with observations on *Mytilus* species grown on-bottom (Coe 1946; Seed 1968; Richardson and Seed 1990), off-bottom (Lauzon-Guay et al. 2005b), or in a laboratory setting (Alunno-Bruscia et al. 2000, 2001). The shells of mussels in congeneric or mixed species groups became more elongated with increased density. However, there was no significant effect of density on shell shape among mixed species or congeneric groups within each genotype. As observed in other studies (Innes and Bates 1999, Miranda 2004, Penney et al. 2007), *M. edulis* also exhibited a significantly more rounded shell than *M. trossulus* at all densities.

A previous study of shell length growth in *M. edulis* and *M. trossulus* at the same study site recorded a 10 or 6mm difference in growth between species at a density of 70 and 100 mussels per pearl net respectively (Miranda 2004; chapter 3). In the present experiment, these differences correspond to the low to medium densities i.e.~15- 30 mussels per 500cm^3 cage. Moreover, in agreement with the high density effect in the current experiment (~60 mussels per 500cm^3 cage), several studies of mussels in suspended culture at high density have also reported no apparent differences in shell length growth between *M. edulis* and *M. trossulus* (Mallet and Carver 1995; Penney et al. 2002, 2006). Previous studies, however, have not examined the effect of very low density on the growth of the two species (Mallet and Carver 1995; Penney et al. 2002; Miranda 2004; Penney et al. 2006). At very low densities, during gap formation for example (Seed and Suchanek 1992), access to space or food (if limiting) is increased, potentially leaving more resources available for individuals to allocate to soma, maintenance, or reproduction (chapter 3). In the current experiment decreasing density revealed large

interspecific differences in allocation of energy to shell growth. These findings are consistent with those from a companion experiment, in which *M. edulis* prioritized allocation to somatic traits and *M. trossulus* prioritized allocation to reproductive traits earlier in life (chapter 3). Moreover, because *M. trossulus* deployed in parallel to this experiment allocated more resources to reproduction than *M. edulis*, the relative absence of somatic allocation with decreasing density in *M. trossulus* compared with *M. edulis* could be attributable to the diversion of a greater proportion of resources to reproductive investment (chapters 2, 3).

Central to the co-existence of *M. edulis* with *M. trossulus* and the likelihood of displacement is their performance in mixed aggregations. Owing to its greater shell growth in the presence of *M. trossulus* than in its absence, *M. edulis* grown with *M. trossulus* is likely less susceptible to predation (Seed and Brown 1978; Seed and Suchanek 1992) or interference competition (Harger 1968; Seed and Suchanek 1992). Compared with *M. trossulus* only, the shell growth of *M. trossulus* in the presence of the much larger *M. edulis* was slightly depressed at the highest densities, while mortality was unaffected. A smaller body size could leave *M. trossulus* more susceptible to predation than *M. edulis* (Seed and Suchanek 1992; Seed and Brown 1978), especially when coupled with a thinner shell or weaker adductor muscle (Hancock 1965; Caro and Castilla 2004; Freeman and Byers 2006; Freeman 2007; Leonard et al. 1999; Reimer and Harms-Ringdahl 2001; Smith and Jennings 2000; chapters 3-4). Finally, it is not clear whether or not the decreased growth of *M. trossulus* grown with *M. edulis* at higher densities is attributable to inhibition by *M. edulis*, potentially reducing the fitness of *M. trossulus*. Because *M. trossulus* exhibits flexible allocation for reproduction according to resource availability (chapter 3) or during starvation (pers. obs.), it is also possible that reproductive character displacement (i.e. enhanced reproduction) could minimize the fitness cost of any interference competition from *M. edulis* making it more difficult to detect. It is also surprising that *M. edulis* mixed with *M. trossulus* increased in body size, unlike *M. edulis* at all densities in the absence of *M. trossulus*. The absence of any

difference among the slopes of *M. edulis* is difficult to interpret strictly in terms of competitive interactions among the two species. Experiments analyzing competitive interactions among species at 1:1 ratios over a range of densities can be too conservative as they contain a mixture of intra and inter-specific effects (Peter Petraitis *pers. comm.*). The finding of no differences in slopes among the *M. edulis* groups could be a consequence of the conservative nature of this test, thereby masking any additional benefits (in terms of growth) for *M. edulis* where it suppresses the growth of *M. trossulus* at higher densities. Further work deploying *M. edulis* and *M. trossulus* at a number of different ratios, ranging from pure *M. edulis* to pure *M. trossulus,* among the different densities is required to resolve this. Overall though, the faster growth of *M. edulis* mixed with *M. trossulus* would allow it to occupy a greater proportion of the available space leaving less room for the slower growing *M. trossulus* to grow (Petraitis 1995).

In conclusion, the co-existence of *M. trossulus* and *M. edulis* likely hinges on the greater reproductive allocation of *M. trossulus* earlier in life (Miranda 2004; Chapters 2-3), the prevailing disturbance regime (Petraitis et al. 1989; Chesson 2000; Shea et al. 2004) and the resultant ratio and density of intra- or inter-specific aggregations. By highlighting large differences between *M. edulis* or *M. trossulus* density dependent growth, the transition from low density to high density is particularly important. Immediately following gap formation, mussel density will be reduced. As mussels continue to grow, in and around a settled gap, mussels will gradually be more strongly regulated by density dependent interactions (Petraitis 1995). The greater reproductive allocation of *M. trossulus* relative to *M. edulis* enhances its ability to recolonize gaps or patches in mussel beds (Toro et al. 2002; Kneitel and Chase 2004; Miranda 2004; Liu 2007; chapter 3) thereby enhancing resilience to disturbance (Southwood 1977). *M. edulis* on the other hand prioritizes allocation to much faster growth or defense if predators are present. This in turn increase the likelihood of *M. edulis* displacing *M. trossulus* especially since the current experiment highlights that the faster growing *M. edulis* can potentially suppress the growth of *M. trossulus* leaving it relatively more

susceptible to predation by crabs. Overall though, together with a disturbance regime that prevents the dominance of *M. edulis* by opening up patches for colonization by *M. trossulus*, differences among *M. edulis* and *M. trossulus* colonization or sucessional abilities can theorectically permit their co-existence (Levin 1974; Tilman 1990; Chesson 2000; Kneitel and Chase 2004; Calcagno et al. 2006, chapter 3).

5.5. References

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Figure 5.1. Example of 500cm^3 cage units housed in pearl-nets.

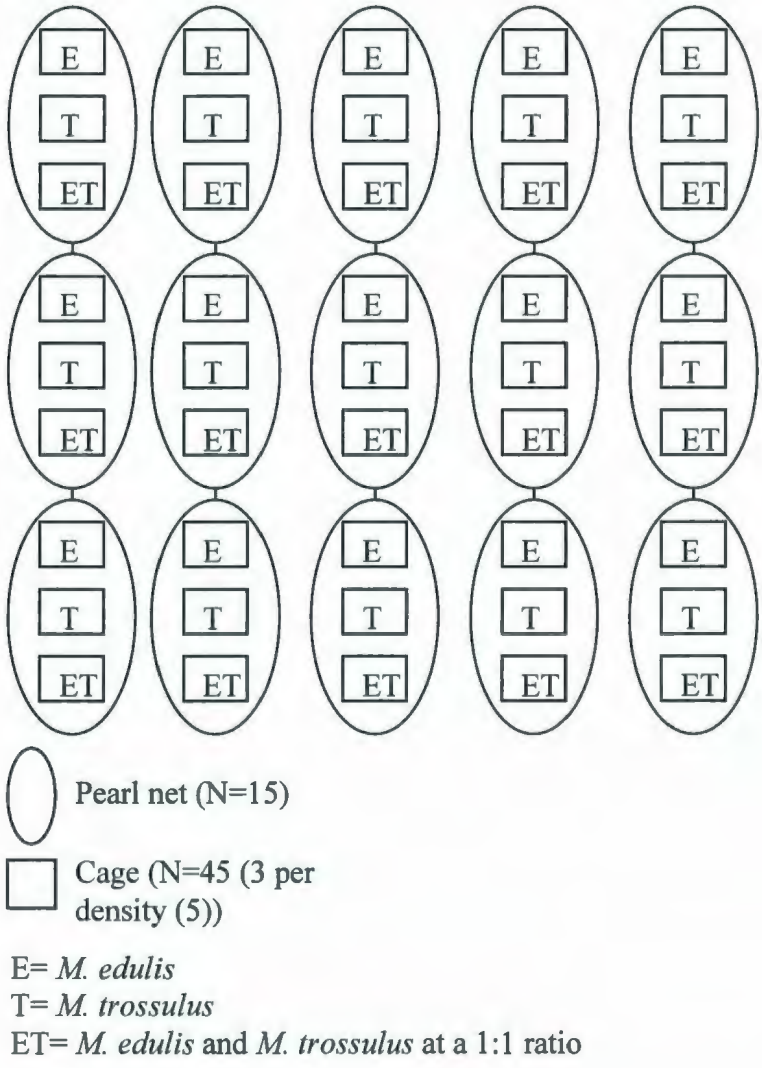
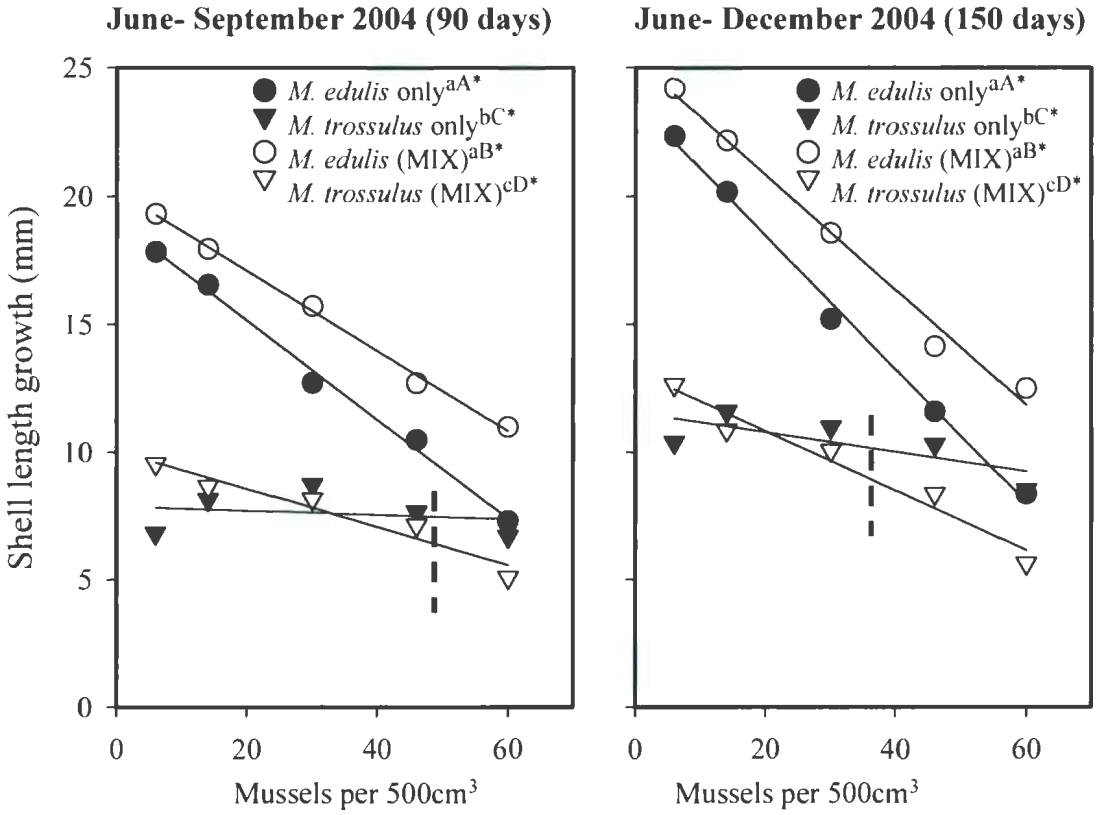


Figure 5.2. Experimental design to compare shell growth of *Mytilus trossulus*, *M. edulis*, *M. edulis* when mixed with *M. trossulus* and *M. trossulus* when mixed with *M. edulis*. Interspecific groups were mixed at a 1:1 ratio in the same cages. Cages of each group at each of 5 densities in triplicate were randomly assigned to 5 strings of pearl nets. The figure shows cages organized into groups of E,T and ET for clarity. Table 5.1 presents the number of mussels for each density.

Table 5.1. The number of mussels (summed across the three cage replicates) for each density.

	Density (no. per 500cm^3 cage)					
	6	14	30	46	60	Sum
Intraspecific						
M.edulis tagged	18	42	45	60	60	225
M. trossulus tagged	18	42	45	60	60	225
M. edulis (tagged plus untagged)	18	42	90	138	180	468
M. trossulus (tagged plus untagged)	18	42	90	138	180	468
1:1 Mixture of each species						
M. edulis tagged	9	21	45	60	60	195
M. trossulus tagged	9	21	45	60	60	195
M. edulis (tagged plus untagged)	9	21	45	69	90	234
M. trossulus (tagged plus untagged)	9	21	45	69	90	234



*Where slopes among groups were heterogenous the Johnson Neyman procedure was used to determine the range of densities where growth was not significantly different among groups. Dashed black line denotes the lowest density where *M. trossulus* groups were significantly different from each other. *M. edulis* "MIX" had significantly greater shell length growth (at 90 or 150 days) than both *M. trossulus* groups at all densities. Pure *M. edulis* shell growth (at 90 or 150 days) was greater than in *M. trossulus* MIX at all densities and greater than pure *M. trossulus* from 6 to 46 mussels per 500cm^3

Figure 5.3. Mean shell length growth (mm) related to density after 90 or 150 days. MIX denotes *M. edulis* or *M. trossulus* in a mixture at a 1:1 ratio with the other species. Regression slopes with the same letter (lower case) were not significantly different. Intercepts with the same letter (upper case) were not significantly different.

Table 5.2. Results of pair-wise contrasts following an ANCOVA of shell length growth data. (unbalanced, Type III Sum Sq., minimal General Linear Model).

Shell length growth (mm) in 2004						
		Slope		Intercept		Range of mussels per 500cm^3 with
	Df	F	P	F	P	significantly different growth
June-September						
E only x E MIX	1,389	4.93	ns	78.46	***	6 to 60 (ALL)
T only x T MIX	1,343	14.00	***	2.06	ns	60 only
E only x T only	1,394	126.54	***	219.57	***	6 to 46
E MIX x T MIX	1,338	18.83	***	530.50	***	6 to 60 (ALL)
June- November						
E only x E MIX	1,397	5.64	ns	100.30	***	6 to 60 (ALL)
T only x T MIX	1,342	19.20	***	25.86	***	46 to 60
E only x T only	1,392	180.37	***	176.32	***	6 to 46
E MIX x T MIX	1,347	29.53	***	651.02	***	6 to 60 (ALL)

*** = P<0.001, ns= non-significant P>0.05

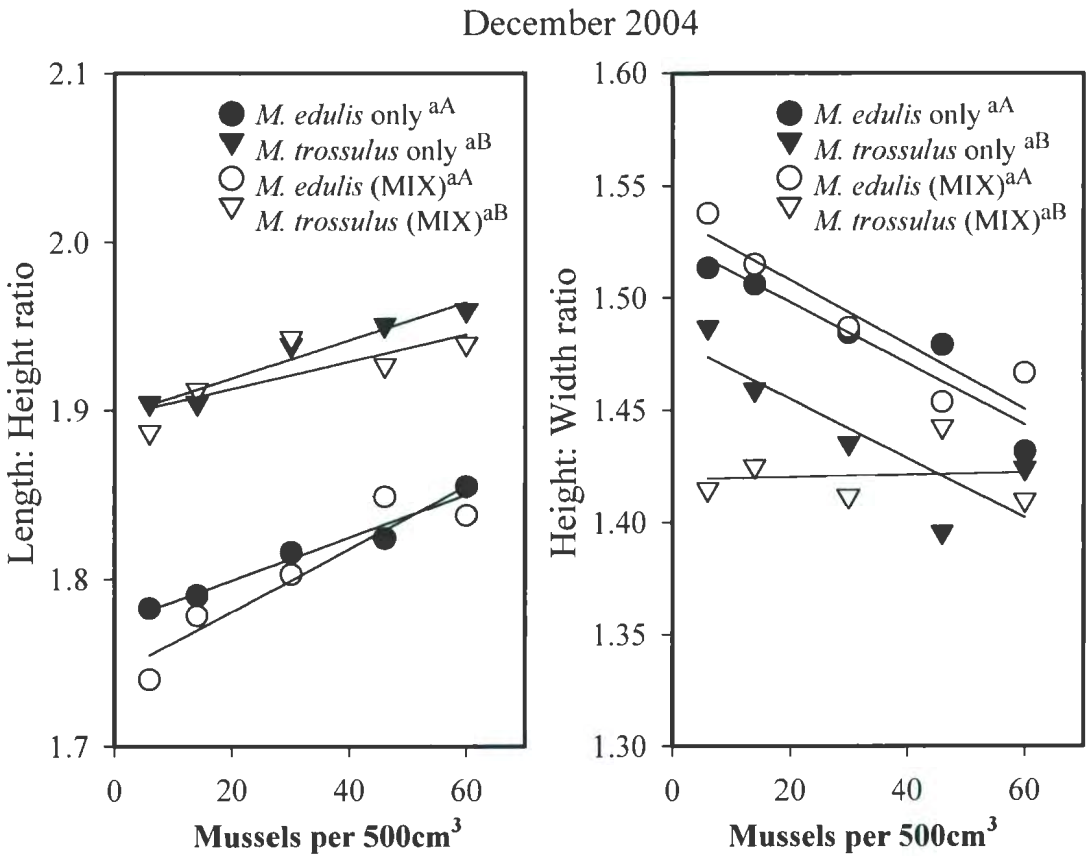


Figure 5.4. Mean shell length: height or height: width ratios in relation to density in December 2004. MIX denotes *M. edulis* or *M. trossulus* in a mixture at a 1:1 ratio with the other species. Regression slopes with the same letter (lower case) were not significantly different. Intercepts with the same letter (upper case) were not significantly different.

Table 5.3. ANCOVA for shell morphology data (unbalanced, Type III Sum Sq., minimal General Linear Model).

Shell morphology

Final length: height ratio (December 2004)

	Df	Sum Sq	Mean Sq	F value	P
Species	1	2.391	2.391	343.2	***
Density	1	0.309	0.309	44.3	***
Residuals	744	5.182	0.007		

Final height: width ratio (December 2004)

	Df	Sum Sq	Mean Sq	F value	P
Species	1	0.430	0.430	54.7	***
Density	1	0.249	0.249	31.6	***
Residuals	744	5.851	0.008		

Main effects
Species: *M. edulis* or *M. trossulus*
Covariate
Density: Mussels per $500cm^3$ (6,14, 30,46,60)
*** = P<0.001

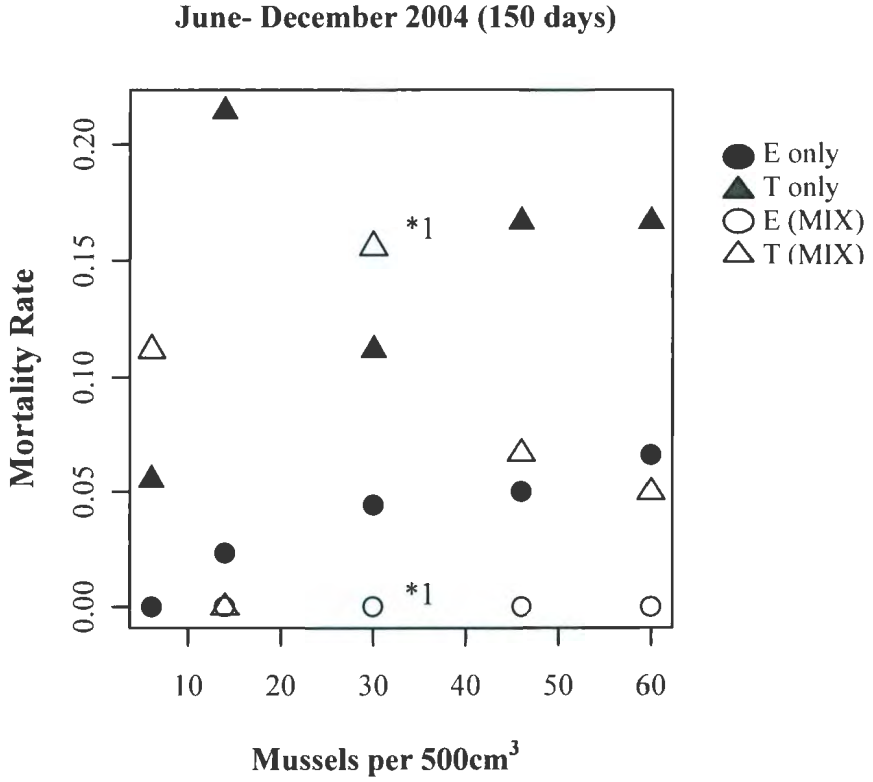


Figure 5.5. Mortality rate for *M. edulis* (E) and *M. trossulus* (T) in relation to density. MIX denotes *M. edulis* or *M. trossulus* in a mixture at a 1:1 ratio with the other species in the same cage. *1. Following contrasts among groups at each density level only E (MIX) and T (MIX) at a density of 30 mussels were significantly different (Chisq = 9.2, df= 1, P= 0.0024). Density also had no significant effect on mortality rate within each group.

Table 5.4. Analysis of Deviance (Generalized Linear Model) for mortality data. a) Effect of density within each group; b) contrasts among groups at each density. MIX denotes *M. edulis* or *M. trossulus* in a mixture at a 1:1 ratio with the other species in the same cage.

a)

Group	Df	Chisq	P
E only	4	2.6204	ns
E MIX	4	2.7268	ns
T only	4	-3E-10	ns
T MIX	4	6.4379	ns

b)

Contrast	Mussels per 500cm^3				
	6	14	30	46	60
E only x E MIX	ns	ns	ns	ns	ns
T only x T MIX	ns	ns	ns	ns	ns
E only x T only	ns	ns	ns	ns	ns
E MIX x T MIX	ns	ns	*1	ns	ns

*1 Chisq 9.2, P= 0.0024

ns =non-significant P>0.05

6. General discussion and conclusions

6.1. General discussion

6.1.1. Allocation patterns and associated ecological differentiation

The optimal allocation of resources to growth, defense, or reproduction and the trade-offs among these traits with survival is a problem affecting all organisms, from viruses to complex multi-cellular plants and animals. The timing and magnitude of energy allocations to various somatic or reproductive functions in fluctuating environments also gives rise to groups of key life-history traits (Stearns 2000). Where differences among life histories arise, this in turn results in ecological differentiation, which reinforces mechanisms that stabilize co-existence of species (see chapters 1, 3). For example, it is generally assumed that the more ecologically distinct that species are, the less likely their physiological, temporal or spatial niches are to overlap (Chesson 2000). Where species are closely related, with similar requirements for a limiting resource, the study of allocation trade-offs among traits represent a useful approach to the problem of how species might compete for a shared resource (chapter 1). Moreover, where closely related species interbreed, divergent life-histories may lead to other pre or post-zygotic barriers to hybridization (Toro 1999; Miranda 2004; Bierne et al. 2006; Liu 2007), for example by establishing successional dynamics or spatial segregation that lead to intra- rather than inter-specific interactions (Chesson 2000; chapter 3), or by creating phenotypic barriers to hybridization (e.g. difference in egg size [Levitan 2006]). Furthermore, the viability of hybrids and the likelihood of "establishment success" (Dieckmann 1997; Dieckmann and Doebeli 1999, 2000, 2003) may be correlated with the hybrids' life-history and the resultant ecological differentiation from the parental species. In this context, interbreeding Mytilus species competing for space and other shared resources provide a useful model for investigating whether or not allocation trade-offs and the resultant plasticity of allocation to traits associated with growth, defense,

reproduction, and survival can help to explain temporal and spatial variation in the abundance of hybridizing species.

Quantifying reproductive allocation in *M. edulis*, *M. trossulus* and their F1 hybrids required an understanding of the gametogenic cycle (chapter 2). Generally mussels within and among the genotypes attained peak reproductive development in synchrony. *M. trossulus* had a greater reproductive output earlier in life than *M. edulis* (chapter 2) and produced smaller eggs. Allocation strategies were established for *M. trossulus*, *M. edulis* and their F1 hybrids after comparing variation among genotypes in plasticity of growth, reproductive effort and survival under various conditions of resource availability (chapter 3). *M. edulis* prioritized allocation to growth, whereas *M. trossulus* prioritized reproduction. At Long Harbour compared with Trinity, both hybrid groups increased reproductive effort, albeit to a much lesser extent than *M. trossulus*, and maintained growth constant. Survival was lowest in hybrid *edulis*, and the survival of hybrid *trossulus* was not significantly different from that of either species.

Experiments on the plasticity of allocation to predator induced defenses showed that both species could recognize and respond to predator cues from crabs or sea stars by enhancing byssal attachment strength (chapter 4). There were also differences between species in shell thickness and adductor muscle mass. *M. edulis* developed larger adductor muscles and thicker shells than *M. trossulus* following exposure to sea-stars and thicker shells than *M. trossulus* in the presence of crabs. The magnitude of these differences suggests that *M. trossulus* is more susceptible than *M. edulis* to predation from crabs or sea-stars, both of which are key predators of Mytilus species. Where the two species co-occur, predator-prey interactions are therefore likely to be important determinants of their temporal and/or spatial abundance.

Increasing density inhibited shell growth more strongly in *M. edulis* than in *M. trossulus*, and at the highest densities growth in the two species was similar (chapter 5).

Given that the availability of space or limiting resources decreases with increasing density for a given area (Seed and Suchanek 1992; chapter 5), the large increase in *M. edulis* growth with decreasing density provides further evidence that *M. edulis* prioritizes allocation to growth. However, when the two species were mixed *M. edulis* grew slightly faster than it did in the absence of *M. trossulus*, and much faster than the *M. trossulus* present in the mixture. At the same time, the presence of *M. edulis* suppressed *M. trossulus* growth at the highest densities, but not its survival (chapter 5).

6.1.2. Adaptive significance of the findings

The combined findings of chapters 2-5 help to lay the foundations for an improved understanding of the distribution, abundance and potential of *M. edulis* and *M. trossulus* to coexist. The prioritization of somatic traits (growth or defense) could explain the increased frequency of *M. edulis* relative to *M. trossulus* with decreasing size class observed at a large number of sites across Newfoundland by Comesaña et al. (1999) and Miranda (2004). Prioritizing allocation to reproduction earlier in life, could explain why a greater proportion of *M. trossulus* larvae settles at the majority of sampled sites (Comesaña et al. 1999; Toro et al. 2004; Miranda 2004). Moreover, *M. edulis* is more likely to succeed established populations of *M. trossulus* following episodes of predation or other forms of disturbance where the thinner shelled constitution of *M. trossulus* leaves it more susceptible. Conversely, increasing reproductive investment earlier in life likely increases the ability of *M. trossulus* to re-colonize bare patches following more indiscriminate episodes of disturbance e.g. post spawning mortality, intense storms, less selective predation, and ice-scouring (chapter 3). Trade-offs among allocation to colonization or successional abilities, together with disturbance regimes, establish the successional dynamics that directly stabilize co-existence (Petraitis et al. 1989; Chesson 2000; Calcagno et al. 2006) or play an important role alongside other stabilizing mechanisms (Chesson 2000; Yu and Wilson 2001; Levine and Rees 2002; Amarasekare 2003; Turnball et al. 2004; Shwillk and Ackerly 2005). The manifestation of patches in a

variety of successional states could then help to explain the patchy spatial and temporal distribution of the two species within Newfoundland. Furthermore, differences in their colonization or "successional" abilities should increase with increased resource availability e.g. following "gap formation", where crowding is reduced, or periods of food abundance (chapters 3, 5). The reduced flexibility of allocation to growth or reproduction of both F1 hybrids relative to *M. edulis* or *M. trossulus,* together with the lower survival of hybrid *edulis*, could also reduce the invasion fitness of the F1 hybrids (Dieckmann 1997). This conclusion is predicated on the principles that established species or individuals should more strongly inhibit invaders more similar to themselves, and that the number of propagules released and establishment success are correlated (Tilman 2004; Bonsall et al. 2004).

While the observed allocation patterns may be unique to *M. edulis* and *M. trossulus*, allocation trade-offs among ecologically important life-history traits can take many forms (Levins & Culver 1971; see also chapter 3) and are thus important determinants of species diversity and diversification (Bonsall et al. 2004). For example, a trade-off between competitive ability and longevity may explain the coexistence of diverse assemblages of Californian rockfish (*Sebastes*), life expectancies among species ranging from 12 to 200 years (Bonsall and Mangel 2004). Moreover in South Africa, the co-existence of the mytilid *Perna perna* with the recently introduced *Mytilus galloprovincialis* has been attributed to a trade-off between attachment strength on one hand and fast growth combined with high reproduction on the other, allowing the relatively more strongly attached *Perna perna* to dominate wave exposed environments (Erlandson et al. 2006). While there is considerable scope to expand our understanding of the co-existence of interbreeding Mytilus species by quantifying allocation to ecologically important traits under fluctuating abiotic or biotic selection pressures, some caution should be exercised when attempting to quantify allocation patterns in *Mytilus*. Ideally, cohorts of known age and size should be used to detect potential allocation trade-offs. Moreover, despite the striking differences in the allocation "tactics" of *M. edulis*, *M.*

trossulus and their F1 hybrids, there remain a number of important caveats that require further attention.

6.1.3. Caveats

6.1.3.1 Perceived risk of mortality

One of the simplest ways to explain the divergent allocation patterns is the perceived risk of mortality (Stearns 2000 and citations therein). Reproduction and maturation earlier in life are often associated with a greater risk of juvenile mortality. Accordingly, it would strongly reduce an organism's fitness if it invested in somatic traits only to die before reproducing. Why the perceived risk of mortality should be greater for *M. trossulus* than *M. edulis* is currently not well understood. Two explanations involving lineage specific constraints or character displacement require further investigation. For example, if the risk of death from indiscriminate disturbance is greater than predation, selection for inducible defenses may be inhibited (Reimer and Harms-Ringdahl 2001). Moreover, character displacement induced by physical contact with, or waterborne cues from, *M. edulis* could limit negative inter-specific interactions by reducing ecological similarity.

6.1.3.2. Prevailing disturbance regime

The prevailing disturbance regime around Newfoundland is critical to the likelihood of the species co-existing through successional dynamics (Petraitis et al. 1989), but unfortunately it is not well understood. Disturbance events (leading to death) arising from predation, spawning, ice scouring and storms are likely. The frequency and intensity of these events, and ultimately the ratio of indiscriminate to discriminate disturbance, are unknown. Simply put, if there is too much disturbance longer-lived species cannot persist in the system; if too little, competitive superiors drive pioneer

species to extinction (Shea et al. 2004). Only at intermediate levels of disturbance can both types coexist. Moreover, the relative susceptibility of each mussel species to wave-action should also be determined. A more rounded shell in Mytilus species increases drag, hydrodynamic load and consequently susceptibility to dislodgment through wave action (Seed and Suchanek 1992). Given that the two species have similar attachment strength when they are not spawning and that *M. edulis* has a more rounded shell than *M. trossulus*, *M. edulis* is likely the more susceptible to wave action during the fall and winter when storms are more frequent.

6.1.3.3. Reproduction and associated fitness

Increased reproductive investment can offset any decreases in fitness associated with reduced growth or defensive allocation. In particular, the fitness cost associated with the relatively slower growth of *M. trossulus* when grown at high density with *M. edulis* could be offset by increased reproductive investment. Unfortunately, due to logistical constraints it was not possible to quantify the reproductive investment of *M. edulis* or *M. trossulus* after exposure to crabs or sea-stars or with increasing density in intra or inter-specific aggregations (chapters 4, 5). No studies on co-existing Mytilus species have examined survival and the allocation of resources to reproduction and growth with increasing density, despite their importance in determining invasion fitness.

6.1.3.4. Resource availability and inducible defenses

Increasing food availability increases the resources available for growth, defense, maintenance, and reproduction. Measuring inducible defenses in response to higher food availability in the field or the laboratory would determine whether or not *M. trossulus* can allocate energy to defensive traits once the requirements of the other allocations have been met.

6.1.3.5. Variation between individuals

Despite strongly significant differences between *M. edulis* and *M. trossulus* mean adductor muscle mass, shell thickness, growth and reproductive effort; there were *M. edulis* and *M. trossulus* individuals in the samples with overlapping values for these traits. Unfortunately allocation to soma, growth, defense and reproduction could not be assessed at the individual level and requires further attention. Individual level allocation patterns may deepen our understanding of the potential invasion fitness of a range of phenotypes within each species (Diekmann 1997; Stearns 2000).

6.1.3.6. Aggregation behavior

Mussel beds are dynamic structures composed of constantly re-arranging individuals (Seed and Suchanek 1992). Position or crawling behavior of mussels within clumps was not quantified in the present study and could yield valuable information on the potential of *M. edulis* to "smother" *M. trossulus.* Further studies on position of mussels within clumps could also determine the potential for *M. trossulus* to crawl on top of *M. edulis*, thereby increasing the height of a clump, leaving it more susceptible to dislodgment by wave action (Seed and Suchanek 1992). The latter behavior, which has been observed in mixtures of *M. californianus* and *M. trossulus* (Seed and Suchanek 1992), would help to reset the successional process (see above 6.1.3.2). Another form of clumping behavior that requires further attention is whether or not individual *M. edulis* or *M. trossulus* preferentially aggregate with conspecifics in the wild. If so, the likelihood of hybridization is decreased, especially if individuals co-ordinate spawning. No evidence of individuals of each species clumping with their conspecifics was obtained in preliminary observations in the laboratory in this study (see also Stapleton 2007). Moreover, mussels on the periphery of a bed are more easily predated than mussels at the center, while predators may induce mussels to clump together more tightly. Preliminary work on the

latter suggested that sea stars and crabs induced tighter clumping in both mussel species although *M. trossulus* clumps were not as tightly aggregated.

6.1.3.7. Egg size and number

Reproductive effort and output estimates were based on the gonad weight of males and females (chapter 2). As female *M. trossulus* spawn smaller eggs, which further enhance its fecundity, the reproductive effort and gamete weight estimates pooled for both males and females in chapters 2-3 are a more conservative estimate of the relative potential of the species to colonize new environments. Further work though is required to determine whether the inter-specific differences in egg size are reflected in differences in larval viability and hence colonization success. One explanation for spawning eggs of a different size to those of a sympatric species is that it may prevent inter-breeding. Where closely related species coexist, sperm from species spawning smaller eggs may not penetrate the "jelly coats" of the larger eggs of other species as efficiently as they do eggs of their own species (Levitan 2006). With these caveats in mind the general conclusions are given in section 6.2.

6.2. General Conclusions

6.2.1. Reproductive trends (chapter 2)

The reproductive cycles were generally similar among species and F1 hybrids, although one instance of a difference between *M. edulis* and *M. trossulus* spawning time was detected at Long Harbour in the second cohort consisting of older mussels. Monthly sampling during the spawning period may not be enough to detect differences in the timing of spawning. Both the peak and decline in the body condition and gonado-somatic indices were greater for *M. trossulus* compared with *M. edulis*. Gamete output, standardized by body-size, was also significantly higher in *M. trossulus* than in *M. edulis*, hybrid *edulis* and hybrid *trossulus*. These gamete output estimates were also integrated

with somatic soft tissue investment values to permit estimation of reproductive effort (chapter 3).

6.2.2. Allocation priorities in hybridizing *Mytilus* spp. (chapter 3)

M. edulis prioritizes allocation to somatic traits early in life to a greater extent than *M. trossulus* (see for example growth trajectories in Figure 6.1). Conversely, *M. trossulus* prioritizes allocation to reproductive traits earlier in life. Like *M. trossulus*, but not *M. edulis*, growth in both hybrid groups did not change with variation in site productivity i.e. no flexible allocation to growth was evident. Some flexibility in reproductive effort was measured in both hybrid groups in response to variation in site productivity, but relative to *M. trossulus* this was small. Survival was also not significantly different between hybrid groups, although survival was lower in hybrid *edulis* than in both species while hybrid *trossulus* survival was not significantly different from that of the parent species. Combined, these attributes reduce the likelihood of F1 hybrid groups co-existing with the parent species through differences in allocation traits associated with colonization or successional abilities. Differences between *M. edulis* and *M. trossulus* in the allocation of resources to ecologically important traits may help to stabilize their co-existence while minimizing fitness differences between the two species (chapter 3).

6.2.3. Predator inducible defenses (chapter 4)

Both *M. edulis* and *M. trossulus* recognized cues from predators, although the magnitude of the response to these cues varied with the predator (sea-star or crab) and the traits measured (growth, attachment strength, shell thickness and adductor muscle mass). Only sea stars affected the growth of both species, which greatly decreased following exposure of mussels to this predator. Predators induced an increase in attachment strength in both species (*M. edulis* > *M. trossulus*). *M. edulis* displayed plasticity in partitioning resources among key defensive traits in the presence of sea stars (growth in adductor

muscle) or crabs (increased thickness of shell). These responses were not observed in *M. trossulus*, which is therefore likely to be more susceptible to invertebrate predation than *M. edulis*. Furthermore, inter-specific differences in mussel shell thickness in the wild could be explained by exposure to crabs. Predation, then, is likely an important source of selective mortality that may partially explain the decreased frequency of larger *M. trossulus* relative to *M. edulis*, thereby increasing the potential of *M. edulis* to succeed *M. trossulus* in mussel aggregations.

6.2.4. Density-dependent growth and shell morphology (chapter 5)

Growth in body-size decreased at a much greater rate with increasing density in *M. edulis* than in *M. trossulus*. Given that space or limiting resources decreases with increasing density, these findings support the argument that *M. edulis* prioritizes allocation to growth rather than reproduction to a greater extent than *M. trossulus*. *M. edulis* also grew to a larger body-size in the presence of *M. trossulus* than in its absence. Conversely, *M. trossulus* growth decreased, albeit weakly, at the highest densities in the presence of *M. edulis*. Together with the fact that survival of individuals in conspecific or inter-specific mixtures of the two species was not strongly affected by density, these findings further emphasize the importance of predation as a key source of disturbance in this system. Moreover, differences in growth between *M. edulis* and *M. trossulus* may be difficult to detect at higher densities. Furthermore, shells became more elongated as density increased, although *M. edulis* maintained a more rounded shell shape than *M. trossulus* at all densities.

6.2.5. General conclusion

To date, few studies have directly compared resource allocation in closely related Mytilus species co-existing in variable environments. Indeed, such comparisons may provide insight into the nature of reproductive costs and the plasticity of allocation that allows different life-history strategies to co-exist in fluctuating abiotic and biotic

environments. Although Mytilus species are amongst the most well studied of intertidal organisms, the recent discovery of sibling Mytilus species co-existing in distinct hybrid zones has raised important questions as to how sessile species with overlapping physiological, space and other resource niches might co-exist and continue to interbreed. Faster growth in the presence of either *M. trossulus* or increasing food availability and/ or space, and enhanced defenses in the presence of predators, increases *M. edulis* resistance to biotic disturbance and therefore its likelihood of displacing *M. trossulus* (chapters 3-5). Conversely, relative to *M. edulis*, *M. trossulus* enhances its reproductive allocation, which likely enhances its ability to re-colonize disturbed environments (chapters 2-3). Differences among *M. edulis* or *M. trossulus* colonization or displacement abilities in disturbed environments in turn reinforce stabilizing and equalizing mechanisms pertaining to their co-existence (chapter 3). Resource allocation trade-offs and associated ecological differentiation in disturbed environments have the potential to play an important role in maintaining species diversity among assemblages of closely related interbreeding sessile species.

6.3. References

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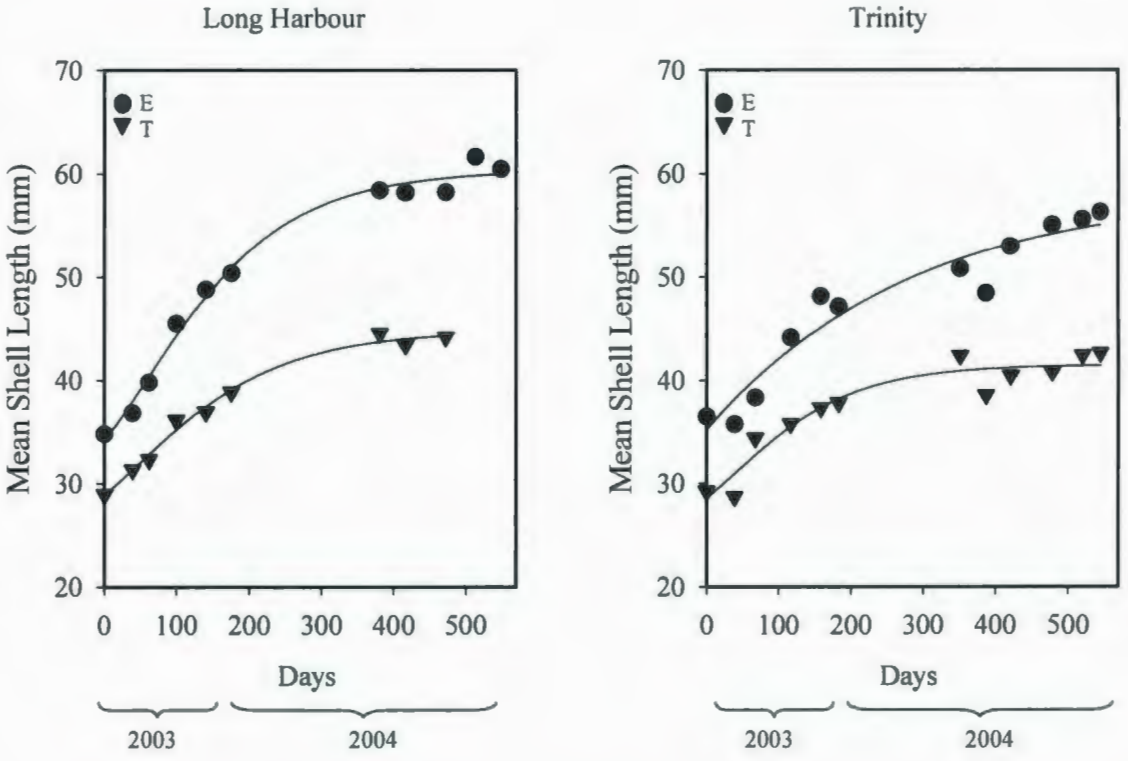

Figure 6.1. Shell length of *M. edulis* (E) and *M. trossulus* (T) during a critical period of divergence in their allocation strategies at Long Harbour and Trinity (Regression lines were fitted by Gompertz growth models). Peak spawning occurred at approximately 70 days and 380 days in 2003 and 2004 respectively at both sites.

7. Appendices

7.1. Calculations for allocation to somatic traits

a) Calculation of initial size (shell length).

Data in equations 1 and 2 were derived from an *Mytilus edulis* mussel (No.1) sampled from Long Harbour at the end of the first deployment period.

Equation 1

Shell length or increment shell length growth

Increment growth (mm)=	B) Distance from notch to shell lip at time t+1…t+x	-	A) Distance from notch to shell lip at initial time x= 0 = 10mm
Increment growth 16.8 mm (May-Dec-03)	= 3) 26.8mm in Dec-03	-	A) 10mm in May

Equation 2

Initial shell length at time t= 0 (start-size)

Initial length =	L) Length at time t+1….t+x	-	Increment growth (May-Dec-03)
Initial length = 35.21mm in May-03	L) 52mm in Dec -03	-	i=16.8mm increment

b) Comparisons of final somatic soft tissue weight adjusted to mussels of standard size

To compare somatic soft tissue growth among genotypes, deployed to Trinity and Long Harbour, the relationship between shell length and somatic soft tissue weight was first determined at the beginning of each sampling period by Ordinary Least Squared (OLS) regression for each genotype x site combination. Since log y =log a +b log x, estimation of an individuals initial somatic soft tissue body weight (y) was possible after substituting an individual's initial shell length (x). The relationship between initial somatic body weight and final somatic body weight was then determined at the end of each sampling period for all genotypes using OLS regression. Comparisons of final somatic soft tissue weight (ln x+1 transformed) for each genotype X site combination were made using ANCOVA with ln x+1 transformed initial somatic soft tissue weight as the covariate. For graphical presentation final-initial weights, adjusted for a mussel of standard initial somatic soft tissue weight, were converted to an energy estimate (1g dry flesh weight = 21.8 kJ (Bayne and Worrall, 1980)) and plotted as somatic soft tissue growth. The predict function in R was used to obtain the mean and standard error for the final soft tissue weight for a standard initial somatic soft tissue weight.

R source code

1. Relationship between shell length and somatic soft tissue weight for the initial sample for each genotype:

   "lm(log(soma)~log(shell_length),data=LH_genotype_initial_sample)"

2. Estimation of initial somatic soft tissue weight for each individual of each genotype (4) deployed to each site (2) sampled at the end of the experiment (e.g. *M. edulis*, (No.1) deployed to Long Harbour (LH)):

a) Initial shell length of *M. edulis* (No.1)= 35.21mm (see section 7.1a).

b) Relationship between shell length and somatic soft tissue weight (initial sample):

   "*M_edulis*_initial_LH<-lm(log(soma)~log(length),data=LH_*M_edulis*_initial_sample)".

c) Estimate of initial somatic soft tissue weight for *M. edulis* (No.1):

   "predict(*M_edulis*_ initial_LH, data.frame(initiallength=35.21), se=T)".

3. ANCOVA (verified for homogenous slopes):

   "All_genotypes<lm(log(soma)~gencode*Site+ log(initialsoma, data=All_genotypes)".

   For unbalanced sums of squares (CAR package): "Anova (G)".

4. Regression model for each genotype within each site.

   " mLHE<-lm(log(soma)~log(initialsoma), data=LH_edulis)"

5. Final somatic soft tissue weight value:

   "predict(mLHE,data.frame(initialsoma=0.04), se=T)"

c) Comparison of shell surface area growth using the homogeneity of slopes model

Using linear regression, the relationship between somatic soft tissue production and time was determined for all genotypes deployed to Trinity and Long Harbour in 2004, while the relationship between shell surface area and time was determined for all genotypes deployed to Trinity and Long Harbour in 2003. Since the slope of each regression for each genotype deployed to either Trinity or Long Harbour describes the rate of change in each somatic trait with time (i.e. growth rate); growth rates were compared by ANCOVA (step 1 below).

R source code

1. ANCOVA (to test the null hypothesis of homogeneous of slopes):
   "G<-lm(soma~daysin*gencode*Site, data= All_genotypes)".
   Summary output for the unbalanced sums of squares (CAR package): "Anova (G)"